Flagellin:allergen fusion proteins as novel vaccines for the treatment of severe type I allergies

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List of abbreviations and acronyms

abbreviation/acronym	explanation
ADAM33	disintegrin and metalloproteinase domain-containing protein 33
AHR	airway hyperreactivity
APC	antigen presenting cell
Ara h 2	*Arachis hypogaea* allergen number 2
B220	protein tyrosine phosphatase, receptor type, C
bp	base pair
Baf A1	bafilomycin A1
Bet v 1	*Betula verrucosa* allergen number 1
BMDC	bone marrow derived denditic cells
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
cDNA	complementary DNA
CD	cluster of differentiation
CD-spectroscopy	circular dichroism spectroscopy
Ci	curie
CpG	oligodeoxynucleotides containing CpG sequence motifs
Cyt D	cytochalasin D
dATP	deoxyadenosine triphosphate
DC	dendritic cell
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
DTT	dithiotreitol
EAE	experimental autoimmune encephalomyelitis
ED_{50}	half maximal release
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FceRI	high-affinity IgE receptor, Fc epsilon RI
FCS	fetal calf serum
Fel d 1	*Felis domesticus* allergen number 1
FITC	fluorescein isothiocyanate
flaA	*Listeria monocytogenes* flagellin A
fliC	*Salmonella typhimurium* flagellin C
Flt-3L	Fms-related tyrosine kinase 3 ligand
Fox p 3	forkhead box protein P3
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEK293	human embryonic kidney cells 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HKL(M)	heat killed *Listeria monocytogenes*
IAA	iodacetamide
IFN-γ	interferon gamma
IgE	immunoglobulin E
IgG	immunoglobulin G
IL	interleukin
IL-4Ra	interleukin 4 receptor antagonist
IMAC	immobilized metal ion affinity chromatography
IPAF	ICE protease-activating factor
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

IPTG	isopropyl β-D-1-thiogalactopyranoside
ISS-ODN	CpG-containing oligodeoxynucleotides
ITIM	immunoreceptor tyrosine-based inhibition motif
KLH	keyhole limpet hemocyanin
L.m.	*Listeria monocytogenes*
LB-medium	lysogeny broth medium
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharides
LRR	leucin rich repeat
mDC	myeloid dendritic cell
M2e	ectodomain of the influenza matrix protein 2
MACS	magnetic cell separation
MHC	major histocompatibility complex
MLN	mesenteric lymph nodes
MOPS	3-(N-morpholino)propanesulfonic acid
MPL®	monophosphoryl lilpid A
MVA	modified vaccinia virus ankara
MVA-Ova	modified vaccinia virus Ankara expressing Ova
MyD88	myeloid differentiation primary response gene (88)
Naip5/Birc1e	neuronal apoptose inhibitory protein
NF	normal food
Ni-NTA	nickel nitrilotriacetic acid
NK cell	natural killer cell
NO	nitric oxide
NOD	nucleotide-binding domain
NP	nuclear protein
OD	optical density
OprI	outer membrane lipoprotein from *Pseudomonas sp.*
Ova	ovalbumin
PAMP	pathogen associated molecular pattern
PBS	phosphate bufferd saline
PBST	PBS Tween
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PLA2	phospholipases A2
PLP-1	encephalitogenic proteolipid protein 1
poly I:C	polyinosinic:polycytidylic acid
PRR	pattern recognition receptor
Pru p 3	*Prunus persica* allergen number 3
R848	resiquimod
rflaA	recombinant *L. monocytogenes* flagellin A
rflaA:Ara h 2	fusion protein containing flaA and Ara h 2
rflaA:Ova	fusion protein containing flaA and Ova
rflaA:Pru p 3	fusion protein containing flaA and Pru p 3
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SipC	*Salmonella enterica* pathogenicity island 1 effector protein
SIT	specific immunotherapy
S-layer	surface layer
SLS	sarcosyl lauryl sulfate

STFΔ2	*Salmonella typhimurium flagellin C* without hypervariable region
ß-ME	beta mercapto ethanol
STAT6	signal transducer and activator of transcription 6
TGF-ß	transforming growth factor beta
Th (1/2/17)	helper (1/2/17) T cell
TIR	toll/IL-1 receptor
TLR	toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
Tr1	regulatory T cell subset 1
T_{reg}	regulatory T cell
Trif	TIR-domain-containing adapter-inducing interferon-β
V	Volt

Acknowledgements

1 Introduction

1.1 Pathomechanism of type I allergic diseases

The prevalence of allergies, especially of inhalant and food allergies is increasing on a world wide scale (1-3). Food allergies are a major cause of long term medical costs and cause a dramatic decrease in quality of life for allergic patients. For example in allergic individuals accidental uptake of µg amounts of allergens, such as the major peach allergen Pru p 3 and the major peanut allergen Ara h 2 may induce severe to fatal clinical reactions (4). Epidemiologic studies suggest that 1.0% to 10.8% of the overall population suffer from food hypersensitivities (5). In industrialized countries the most common form of allergies, IgE-mediated hypersensitivity was reported to affect more than 25% of the population (6,7).

Onset and maintenance of type I allergies are caused by exaggerated Th2-mediated immune responses directed against otherwise harmless antigens. The induction of immune responses is tightly controlled by antigen presenting cells (APC) such as dendritic cells, monocytes, macrophages, and B cells (8). Among the different APC subtypes dendritic cells (DC) are the most potent APC since they are capable of both inducing and regulating immune responses (9-11). DC reside in virtually all peripheral tissues where they continuously sample the repertoire of peripheral proteins for potentially hazardous agents by internalizing proteins specifically and unspecifically. These proteins are further on processed by the proteasome into peptides and loaded onto MHC class II molecules (8). Upon uptake of potentially foreign antigens and activation of the DC by danger signals, the DC loses the ability to internalize antigens, starts to express the co-stimulatory molecules necessary to induce T cell activation, leaves the peripheral organs, and migrates via the lymphatics to draining lymphoid organs. There, antigen loaded MHC II molecules are presented on the APC surface to recirculating antigen-specific CD4 T cells which recognize the antigen derived peptides in the context of self MHC II molecules (8,12).

The function of these CD4 positive T helper cells lies in directing the induced immune response (8). Hereby, the type of adaptive immune response induced depends on the activation status of the DC. Furthermore, the differentiation of naive antigen-specific T cells into effector T cells is controlled by the cytokine milieu (Figure 1). If naïve T cells are activated in the presence of DC-derived interleukin (IL)-12 this induces their differentiation into Th1 cells which function in the clearance of bacteria and tumors. In contrast to this, the presence of IL-4 results in the induction of Th2 cells which are thought to have their primary function in the elimination of multicellular parasites (Figure 1). Hereby, the source of the IL-4 initially needed for Th2 induction remains to be identified. Additionally, the CD4 positive Th17-subset was shown to have a significant impact in immunobiology. Th17 cell

differentiation requires the cytokines IL-23 (13,14) and IL-6 (15). Recent studies suggest extensive activation of Th17 cells to be the cause for some autoimmune diseases, whereas the natural function of Th17 cells seems to be pathogen clearance in mucosal tissues, (16,17).

Normally, the balance between different T cell subsets is tightly controlled by both intrinsic control mechanisms and regulatory T cells producing immunosuppressive cytokines such as IL-10 and TGF-β, since excessive activation of either Th1, Th2, or Th17 cells is likely to result in pathologic immune activation. Hereby, uncontrolled activation of Th1 and Th17 cells may result in autoimmunity, whereas excessive triggering of Th2 cells results in allergic diseases.

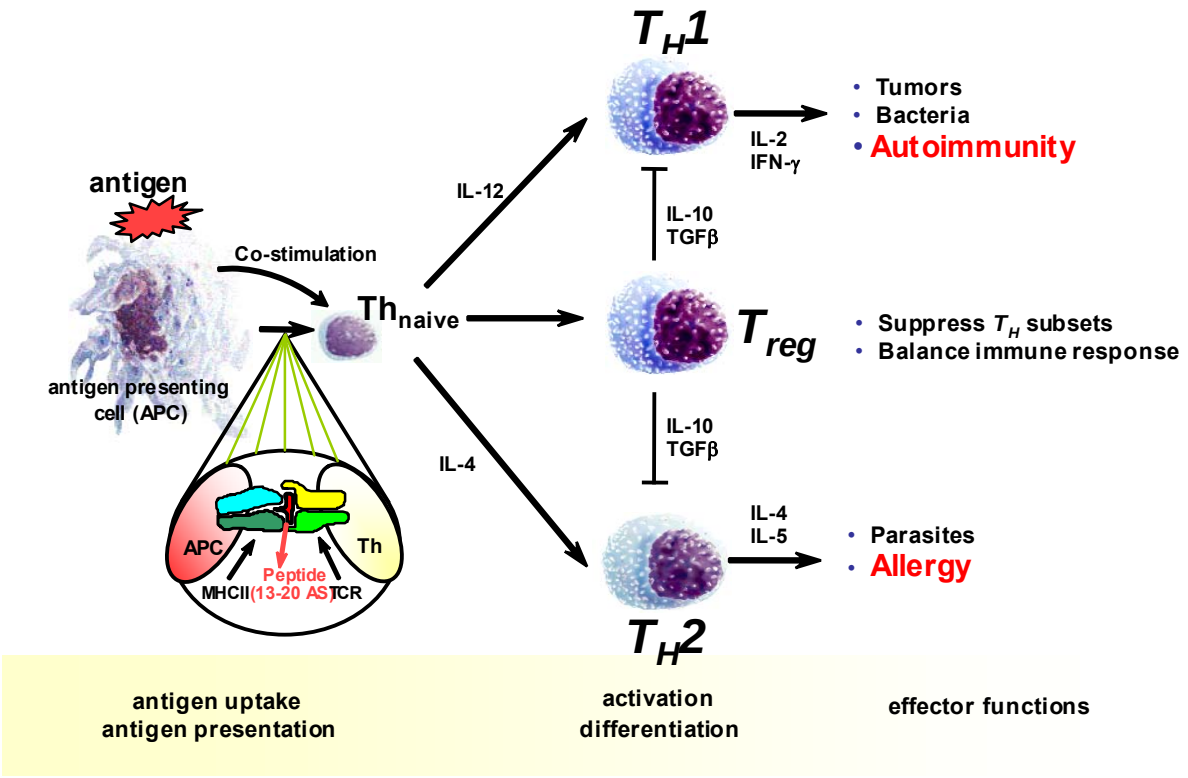


Figure 1: APC induce and control immune responses. For detailed information see text.

During allergic sensitization allergen uptake and processing by antigen presenting cells leads by mechanisms not fully understood to the induction of allergen-specific Th2-cells. These Th2 cells produce the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 which both activate allergen-specific B cells and induce isotype switching resulting in allergen-specific IgE production. Moreover, these cytokines are able to induce maturation of inflammatory cells such as mast cells and eosinophils (8). These cells express the high affinity IgE receptor FcεRI which enables them to bind allergen-specific IgE on their cell surface (Figure 2).

Subsequent second contact with the allergen results in crosslinking of surface bound allergen-specific IgE on mast cells and eosinophils inducing the secretion and de novo

production of pro-inflammatory mediators such as vasoactive amines (e.g. histamine), lipid mediators (prostagladines), chemokines, and cytokines by massive degranulation. These inflammatory mediators then cause the allergic symptoms (8,18,19) (Figure 2).

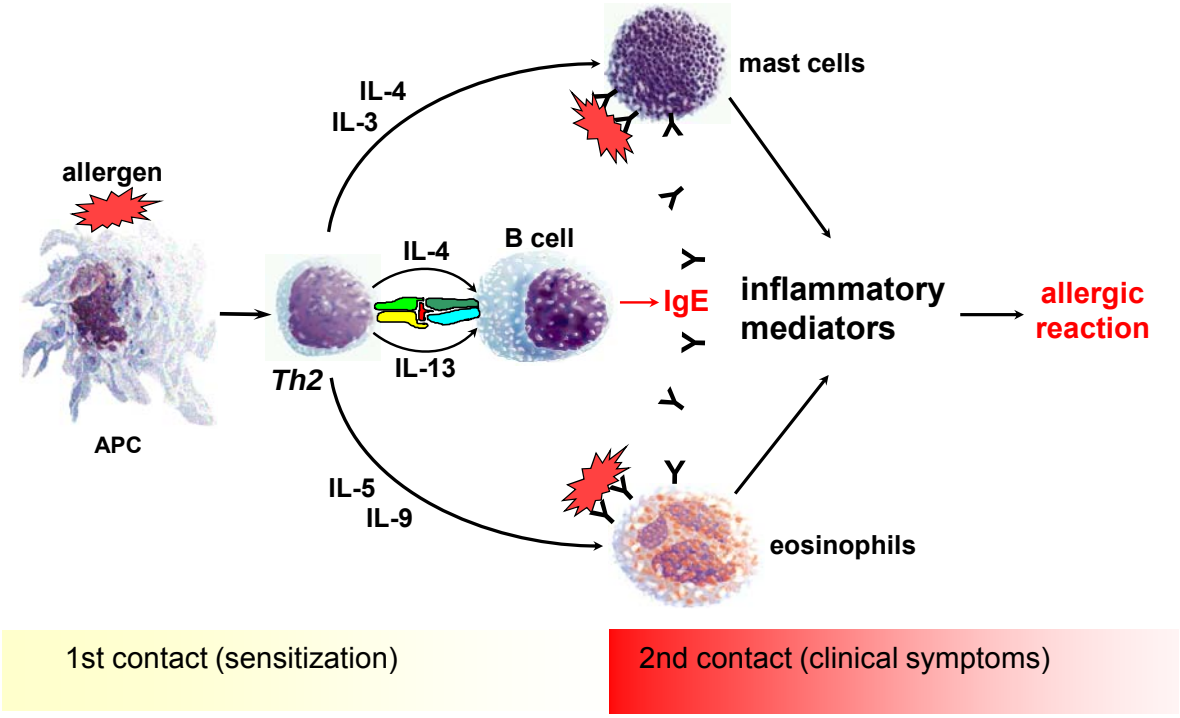


Figure 2: Pathomechanism of type I allergy. For detailed information see text.

The aetiology of allergic immune responses is a complex process influenced by several factors such as genetic susceptibility, environmental factors, route of exposure, dose, and structural characteristics of the allergen (20). Over the last decades much effort has been invested in finding the genes responsible for allergic diseases, since the fact that allergic disorders are more common in persons of West African ancestry compared to persons of European ancestry indicated a certain hereditary component (21,22).

Candidate genes indentified to contribute to allergic diseases can be summarized in groups of genes involved in (i) modulating responses to environmental exposures (e.g. components of the innate immune system that alter the risk of allergic diseases upon microbial co-exposure such as CD14 and TLR4) (ii) the maintenance of epithelial barriers (e. g. filaggrin) (iii) regulating the immune response (among others IL-4Ra, IL-23, STAT6) and therefore Th1/Th2 differentiation and effector function, (iv) determining the tissue response to chronic inflammation in order to induce for example airway remodelling (ADAM33) (reviewed in (20)).

Among those candidates the finding that common mutations in the filaggrin gene are closely linked with ichtyosis vulgaris as well as atopic dermatitis and allergy represent up to date the single most significant breakthrough in understanding the genetic basis of complex allergic diseases (23-26). Filaggrin is an epidermal protein involved in maintaining the skin barrier

and has an impotant function in blocking the entry of microbes or allergens. Therefore, disruption of the skin barrier in patients carrying filaggrin mutations is likely to result in extended and facilitated contact of dermal immune cells (e.g. Langerhans cells) with antigens resulting in a higher risk of developing allergic disorders. Moreover, total IgE-levels in allergic patients, a quantitative trait related to both asthma and allergy, were found to be associated with functional variants of the gene encoding for the alpha chain of the high affinity IgE receptor (27).

However, besides these findings the missing heridability of allergic diseases in many families and the inability to find more candidate genes led to the conclusion that additional factors are likely to influence the development of allergies (28,29). Among those, epigenetic factors certainly play an important role. For example neonates of allergic mothers were shown to display substantial changes in splenic DC DNA methylation, leading to enhanced presentation of allergens by these cells *in vitro* (22,30). Moreover, environmental factors contribute to the pathogenesis of allergies and asthma. These factors are related to age as increasing lifespans result in continous exposure to environmental factors. Concordantly, monocygotic twins born with identical genomic information that develop distinct epigenetic modification patterns, lifestyles, and contacts to environmtal factors are known to differ in their suceptibilty to allergic diseases (31). Therefore, while the immune reactions during acute allergic responses are quite well understood, the mechanisms by which allergies develop, the cells involved, the genetic basis, and why some proteins have the potential to become allergens in susceptible individuals whereas the vast majority of other proteins have no allergenic potential (32) remain largely unknown.

1.2 Current strategies of allergen specific immunotherapy

For the acute (symptomatic) treatment of allergic reactions different medications are available. Among others these include antihistamins, β2-antagonists, adrenaline, cortisol, and in severe cases immunosuppressive drugs such as cyclosporine A (33,34). However, these treatments may only block or decrease allergic symptoms but do not cure the allergic disease.

In 1911 Noon performed the first allergen-specific immunotherapy (SIT) approach using grass pollen extracts (35). Today, nearly 100 years later besides avoiding the allergy causing products the only treatments for allergies are still based on desensitization approaches including the application of increasing dosages of allergen extracts over long periods of up to 3-5 years (36,37). The aim of this allergen-specific immunotherapy is to redirect Th2 dominated immune responses towards either more tolerant or Th1-dominated immune responses by continous exposure to the allergen. In SIT allergen extracts are either applied

subcutaneously or sublingually. Hereby, the well established subcutaneous treatment seems to be slightly more efficient, whereas the more recent sublingual application is more convenient for the patient. While SIT has proven effective for several respiratory and insect venom allergies, for the treatment of food allergies (e.g. peanut allergy) serious side effects are encountered (38). Therefore, currently avoidance of the allergy causing food products is the only possible strategy to cope with severe food allergies.

The problems encountered for the treatment of allergies are mainly caused by the usage of allergen extracts since these extracts are rather crude preparations. Although biological standardisation and potency measurements are well defined, total protein content or the content of single allergens display pronounced batch to batch variations (39,40). Furthermore, extracts may lose their allergen activity during extraction and storage or have a low allergen content (41). Additionally, the inherent enzymatic activity of extracts may lead to protein and especially allergen degradation reducing therapeutic efficacy. Moreover, there are reports showing that allergen extracts may be contaminated with allergens from other sources (42). Taken together, these problems result in unavoidable adverse effects when applying extracts, caused by the highly heterogenous nature of extracts. Consequently, it was proven to be difficult to achieve effective therapeutic dosages using crude extracts in allergic patients. Due to these reasons currently SIT using extracts may not be efficient and be accompanied by a rather high risk of adverse reactions (43). Moreover, due to long treatment periods, SIT is cost-intensive and inconvenient for the patient, leading to a high rate of patients aborting SIT treatments (44). Therefore, all new forms of therapy should aim at increasing efficacy, convenience, and safety for the patient.

Treatment of allergies may either be performed as therapeutic (secondary prevention) or prophylactic (primary prevention) intervention. Currently, different strategies are tested for their therapeutic potential in both mouse models and human allergic patients (see 1.4). Moreover, first prophylactic studies are conducted treating high risk children with bacterial extracts (Wahn, unpublished). Unfortunately, due to the complexity of allergic diseases and the differences between individual patients there is a lack of reliable biomarkers to monitor the success of SIT. Currently, the efficacy of allergy treatments is measured by reductions in the symptom medication score which includes both the occurance of allergic symptoms and the usage of rescue medication. To improve efficacy testing of allergy vaccines, more effort needs to be directed at finding primary endpoints including biomarkers.

1.3 The mode of action of SIT is poorly understood but includes several mechanisms of action

1.3.1 SIT influences many clinical parameters

The overall goal of SIT is to re-establish immunological and clinical tolerance. Hereby, the SIT-induced protective immune responses may be reinforced by natural exposure to the allergen, providing long-term cures for allergies without the need for continuous therapeutic treatment (45). The mechanisms associated with specific immunotherapy are thought to involve changes in both cellular and humoral immune responses. In humans these include a modest reduction in allergen-specific IgE levels accompanied by increases in allergen-specific IgG antibodies, particularly of the IgG4 isotype (46-48). Furthermore, the infiltration of effector cells (mast cells, eosinophils, basophils) and the release of inflammatory mediators were shown to be reduced at the site of allergic inflammation (49-51). Moreover, modulation of T cell responses in conventional allergen-specific immunotherapy were shown very early to be associated with the induction of protective IgG antibodies, as well as antigen-specific regulatory T cells producing IL-10 or TGF-β, or an immune deviation towards more Th1-dominated immune responses (37,50-54). Such immune deviations may be mediated by the induction of Th1 cytokines such as IFN-y suppressing IgE-production and leading to the induction of allergen-specific IgG2a antibodies in mice, suggested to have blocking activities. These properties have been discussed to be at least in part responsible for the beneficial effects mediated by allergen-specific immunotherapy (55).

1.3.2 Modulation of immune responses is tightly controlled by highly specialized cells

In order to understand how SIT might induce the desired immune deviation away from Th2-dominated allergen-specific immune responses the network controlling innate and adaptive immune responses needs to be described in more detail. As mentioned above the induction of immune responses is tightly controlled by antigen presenting cells (APC) such as dendritic cells, monocytes, macrophages, and B cells (8). Among these, dendritic cells (DC) are the most potent APC since they are capable of both inducing and regulating immune responses (9-11). Besides inducing adaptive immune responses by activating antigen-specific T and B cells, DC also control innate immune responses. Innate immunity relies on recognizing conserved pathogen associated molecular patterns (PAMP), highly repetitive structures restricted to pathogens such as bacterial cell wall components. Therefore, innate immunity utilizes conserved receptors which are identical for all members of a given species.

Activation of these receptors results in protection of the host against pathogens during the first days of infection until adaptive immunity is triggered and antigen-specific T and B cells are activated, proliferate, and finally clear the infection. The mechanism by which DC are primarily able to discriminate between dangerous molecules as opposed to harmless self components relies on identifying PAMPs by the means of toll-like receptor (TLR)-engagement (12,56-58). TLR are trans-membrane receptors consisting of an extracellular domain containing leucine rich repeats (LRR) and a cytosolic Toll/IL-1 receptor (TIR) domain (59,60). Ligands for TLRs are highly conserved, mostly repetitive structures restricted to bacteria and viruses (61). Recognition of danger signals such as TLR-ligands by APC induces up-regulation of MHC molecules, production of cytokines, and expression of co-stimulatory molecules. This allows the APC to migrate to lymphoid organs and induce immune responses (62-64). Moreover, TLR engagement induces a gene expression program dedicated to both innate clearance and acquired immunity to pathogenic microorganisms by activating NK cells as well as priming and amplifying T and B cell effector functions, thereby linking innate and adaptive immunity (65,66).

TLR-mediated recognition of PAMP is complemented by the nucleotide-binding domain (NOD), leucine rich repeat containing receptor familliy (NOD-like receptors), consisting of 20 members in mammals (57,58) which detect muramylpeptides released from bacterial peptidoglycan in the cytoplasm. Additionally, retinoic-acid-inducible protein 1 (RIG-I) like receptors sense single stranded viral RNA (67).

While DC are by far the most potent inducers of T helper cell responses (11), DC also play an important role in regulating immune responses by controlling both central and peripheral tolerance and by differentiating regulatory T cell subsets (68,69). While central tolerance leads to the deletion of newly generated, autoreactive T cells in the thymus (8) peripheral tolerance is mediated in the lymphoid organs. Here, contact of immature DC with autoreactive T cells leads to a state of functional T cell unresponsiveness termed anergy. Anergy is induced because immature DC do not express the co-stimulatory molecules required for T cell activation but secrete anti-inflammatory cytokines such as TGF-ß and IL-10 (9,10). If these anergic T cells will further on recognize their specific self-antigen they are not activated any more.

Furthermore, DC control peripheral tolerance by inducing regulatory T cells (T_{reg}) (9,70-72). The most important regulatory T cell subtypes are naturally occouring $CD4^+CD25^+$ T_{reg} and induced Tr1 cells producing IL-10 (73). CD4 CD25 double positive T_{reg} are a naturally occurring T cell subset in the blood and other lymphatic organs characterized by the expression of the fork head transcription factor Fox p 3 and extended regulatory properties (74,75). Lack of T_{reg} by antibody mediated depletion in mice or by naturally occurring mutations (IPEX syndrome) in humans results in strongly enhanced autoimmunity (76-78).

Therefore, T_{reg} induction leading to production of IL-10 and TGF-ß as well as cell-contact dependent suppressor mechanisms is important to maintain both self-tolerance and support clinical efficacy in SIT. T_{reg} may suppress B cell derived IgE production, facilitate IgA and IgG4 production in allergic patients, and were shown to inhibit both production of Th2 cytokines by allergen specific T cells and decrease the recruitment and activation of mast cells, basophils, and eosinophils in mouse models (79). In line with this, murine pulmonary DC were shown to produce IL-10 and mediate tolerance towards respiratory allergens by the induction of T_{reg} (80). Furthermore, in this context sublingual allergen application might be of certain potential since Langerhans-like DC in the oral mucosa are prone to IL-10 and TGF-ß production (81,82).

1.3.3 DC are promising target cells for immunotherapy

After loading with antigen DC exhibit properties of both antigen and adjuvant (9). Moreover, DC display a certain plasticity since DC function can be altered by the local cytokine environment and other factors (9). Therefore, DC are interesting target cells for active immunotherapy referring to the modulation of the immune system for therapeutic purposes (10).

Among the cytokines produced autocrine IL-10 production by many different cell types plays an important role in maintaining immune balance in order to prevent strong immune reactions to inhaled or ingested allergens (83,84). Concordantly, human DC exposed to IL-10 were shown to induce a state of alloantigen-specific anergy in CD4 and CD8 TC by converting DC into a tolerogenic state (85). Additionally, in humans IL-10 treated DC displayed a reduced IL-12 production and were capable of inhibiting allergen-specific Th1 and Th2 cells (86). The T cells induced by IL-10 treated DC also produced IL-10 and exhibited a regulatory phenotype by suppressing peripheral T cell activation in a cell contact dependent manner (86,87). Moreover, DC isolated from IL-10 over-expressing mice displayed an immature phenotype and induced significantly enriched T_{reg} numbers in spleens (68).

Taken together these results showed that targeting and manipulation of DC function might be a tool to significantly increase efficacy and safety of immunotherapy for allergies.

1.4 New strategies for the treatment of allergies

Since there is an increasing need for the development of novel treatments and vaccines especially for allergies, during the last years several novel treatment regimens, application routes, adjuvants, and immune modulators have been investigated. Among others, these include the usage of (1) new delivery routes (intranodal, transcutanous, or sublingual), (2) new formulations and delivery systems (e.g. DNA-vaccination, MVA-based vaccines, or liposomes), (3) recombinantly produced allergens, hypoallergens, and peptide based vaccines, as well as novel adjuvants derived from bacteria and viruses such as (4) bacterial extracts and (5) TLR-ligands.

1.4.1 Recombinant allergens, hypoallergens and peptides

1.4.1.1 Recombinant allergens may be used to improve therapeutic efficacy and safety

To circumvent the problems currently faced by the application of crude allergen extracts recombinantly produced allergen molecules were tested for their therapeutic potential. If the relevant allergens are known and their sequence information is available recombinant allergens can be produced using bacterial or eukaryotic expression systems. The advantages of recombinant allergens over allergen extracts are: (i) very high purity; (ii) generation of defined proteins with known molecular, immunologic, and biological characteristics; (iii) recombinant molecules can be precisely characterized to obtain consistent, reproducible qualities of product batches; (iv) recombinant proteins can be modified to reduce side effects while boosting advantageous immunological properties (39,88). Moreover, single allergen preparations in combination with component resolved diagnosis (usage of naturally purified or recombinantly produced allergens to determine the individual patients sensitization profile) allows selective desensitization approaches, thereby increasing efficacy and safety (89). Additionally, for multisensitized patients mixtures of the relevant purified allergens can be easily generated using the single recombinant allergens (see timothy grass allergen studies discussed later).

Using recombinant DNA techniques recombinant allergens can be produced as molecules exactly mimicking the properties of the natural allergens (recombinant wild-type allergens) or as modified variants with beneficial properties such as reduced allergenic activity and increased immunogenicity (88,90). Moreover, hybrid molecules resembling the epitopes of several different allergens may be generated to include the relevant epitopes of complex allergen sources (90). Naturally occurring isoforms of the major birch pollen allergen Bet v 1 indicated hypoallergenic Bet v 1 isoforms to be potent activators of allergen-specific T

lymphocytes, whereas Bet v 1 isoforms with high IgE-binding activity and allergenicity may display low T cell antigenicity (91). Therefore, treatment with high doses of such hypoallergenic isoforms or recombinant variants of atopic allergens might modulate the quality of the T helper cell response to allergens *in vivo* while reducing the risk of anaphylactic side effects.

In the first larger randomized, double-blind, placebo-controlled clinical study an equimolar mixture of 5 recombinant wildtype timothy grass pollen allergens-Phl p 1, Phl p 2, Phl p 5a, Phl p 5b, and Phl p 6- adsorbed to aluminium hydroxide-was tested in patients with grass pollen allergy (92). In a second double-blind, placebo-controlled multicenter study 147 patients with birch pollen allergy were treated with either the hypoallergenic Bet v 1 variant rBet v 1a, purified natural Bet v 1, birch pollen extract, or a placebo (93). In both studies, actively treated patients showed improved symptom medication scores and developed robust IgG1 and IgG4 antibody titers against natural pollen allergens (92,93), suggesting an increased safety and efficacy compared to treatments using allergen extracts.

1.4.1.2 Hypoallergenic variants hold potential to improve SIT

Next recombinant DNA technology was used to create genetically modified allergens with “hypoallergenic” properties. In order to increase the safety of SIT these engineered allergens for immunotherapy should ideally display both decreased IgE-binding and allergenicity, combined with a conserved or even increased immunogenicity (39). Hereby, hypoallergenic allergen variants should contain all relevant T cell epitopes in order preserve their capacity to induce beneficial IgG antibody responses (6). In line with this, well characterized, hypoallergenic allergen derivatives have been developed for many of the most common allergens (90,94).

Most of the studies investigating the potential use of hypoallergenic variants were performed using the model of birch allergy since the vast majority of birch pollen allergic patients are monosensitized to a single major allergen Bet v 1. Kraft and co-workers generated two adjacent peptides covering the entire Bet v 1a sequence produced in *E. coli*, which contained most of the relevant T cell epitopes, but showed no IgE binding capacity due to a disruption of conformational epitopes. Consequently, these peptides were not able to activate mast cells and basophils derived from sensitized patients (95). In a similar approach a mutated form of Bet v 1 with six point mutations was found to have a strongly reduced reactivity both with serum IgE from birch pollen-allergic patients and in skin prick tests (96). In contrast to this, proliferation assays using Bet v 1-specific T cell clones showed that the mutant retained its T cell activating capacity, necessary for immune modulation (96). Additionally, Bet v 1 trimers were repeatedly shown to display strongly reduced allergenicity in birch allergic

patients determined by skin prick tests (97,98). In line with this Pauli and co-workers showed genetically modified hypoallergenic Bet v 1 derivatives (rBet v 1 fragments, a rBet v 1 dimer, and a rBet v 1 trimer) to have a 100-fold or more reduced capacity to induce immediate type skin reactions in french birch allergic patients (97,99-101). Moreover, in a Swedish patient group vaccination with a recombinant hypoallergenic Bet v 1 trimer strongly induced Bet v 1-specific IgG (IgG_1, IgG_2, and IgG_4) antibody responses while reducing Bet v 1-specific Th2 responses (102). In accordance with the results obtained in human patients, immunization of BALB/c mice with rBet v 1 fragments or a rBet v 1 trimer absorbed to aluminium hydroxide was shown to induce lower IgE but higher IgG1 responses compared to the rBet v 1 wild-type molecule (103). Furthermore, the IgG1 antibodies induced upon administration of both hypoallergenic rBet v 1 derivatives proved to be blocking antibodies that strongly inhibited binding of birch pollen allergic patients' IgE to the unmodified Bet v 1 molecule (103).

Besides using the major birch pollen allergen Bet v 1, several other allergens were modified to generate hypoallergenic molecules for therapeutic purposes. A five-point mutant of the major apple allergen Mal d 1 (104), a mutated form of a ryegrass allergen Lol p 5, (105), and three major peanut allergens, Ara h 1, Ara h 2, and Ara h 3 modified by site-directed mutagenesis (106) were shown to display both strongly reduced capacities to bind specific IgE and reduced reactivities in skin prick tests. Interestingly, all hypoallergens retained the potency to induce T cell proliferation. Moreover, in an elegant approach data obtained by sequential epitope mapping were used to generate a mutant of the major shrimp allergen Pen a 1, VR9-1 which showed a 90 to 98% reduced allergenic potency as determined by rat basophilic leukemia (RBL) mediator release assay both using human and mouse sera (107). In line with these findings, site-directed mutagenesis experiments performed with the major cherry and birch pollen allergens Pru av 1 and Bet v 1 revealed that amino acid S112 was required for IgE binding in the majority of patients sera (108). Finally, introduction of a proline residue in position 111 of the major apple allergen Mal d 1 strongly reduced allergenicity (109).

Taken together, these results suggest that due to a reduced risk of anaphylactic side-effects such hypoallergenic derivatives might be interesting candidates for safer immunotherapy of allergy (110).

1.4.1.3 Peptide based vaccines have a high risk of adverse reactions

To further improve allergen-specific immunotherapy allergen derived peptides have been evaluated as therapeutic agents in allergic diseases. Hereby, peptide design has allowed the generation of peptides containing T cell stimulatory epitopes with the potency to induce protective IgG responses in both mice and humans (51). These peptides retain the potential to influence T cell function, while strongly reducing the risk of anaphylactic reactions requiring IgE-crosslinking by conformational epitopes (51). Allergen-derived peptides can be administered in much larger doses than possible with conventional allergens and may still affect antigen-specific T cells. Upon *in vivo* administration, these peptides may activate allergen-specific CD4 TC in the absence of costimulation which might induce T cell anergy rather than functional TC activation (45). Studies mostly using cat allergen (Fel d 1)-derived peptides have reported a reduction in IL-4 production using either T cell lines (111) or peripheral blood mononuclear cell (PBMC) cultures after allergen stimulation. Furthermore, experiments with human PBMC using multiple peptides have shown decreased proliferative responses and a reduced production of the cytokines IL-4, IL-13, and IFN-y after allergen stimulation (112), accompanied by significant increases in allergen-stimulated IL-10 production.

However, peptide treatments were associated with a high frequency of adverse reactions, possibly caused by peptide-dimers inducing IgE-crosslinking on mast cells or basophils (51).

1.4.2 Bacterial extracts

Since studies showed that exposure to bacterial or viral infections during early childhood may reduce the risk for the development of allergies (“hygiene hypothesis”) (113,114) whole bacteria or bacterial components are considered as potentially interesting vaccine components. Hereby, strategies to improve SIT for severe type I allergies using whole microbes rely on their “intrinsic adjuvant activity”. Here, adjuvant activity is provided by bacterial components (PAMPs) recognized as danger signals by pattern recognition receptors (PRR) on immune cells resulting in immune activation. Bacteria were either administrated as live bacteria (*Chlamydia*, lactic acid bacteria, *Bacillus Calmette Guerin*) (115,116) or heat killed preparations (heat killed *Mycobacteria, E. coli* and *Listeria*) (55,117). The advantage of using heat-killed or otherwise inactivated bacterial preparations over live bacteria lies in reducing the risk of side effects possibly induced by the application of potential pathogens.

1.4.2.1 Heat killed Listeria are potent immune modulators

Among the different bacterial preparations used for immunotherapy heat killed *Listeria monocytogenes* (HKL) were shown to have strong immune modulating capacities. *Listeria monocytogenes* (*L.m.*) is a gram-positive flagellated bacterium causing food-bourne infections in animals and humans (118,119). Vaccination with *L.m.* was shown to result in strong immune responses also directed against passenger antigens such as: ß-galactosidase, Ovalbumin (Ova), influenza nucleoprotein (NP), LCMV NP and tumour antigens (120).

In 1998 Yeung et al. (121) demonstrated that co-administration of HKL and keyhole limpet hemocyanin (KLH) to KLH-sensitized mice was sufficient to reverse established Th2-responses, resulted in the production of Th1-cytokines such as IFN-y and the induction of high titers of KLH-specific IgG2a antibodies whereas both IgE production and Th2 cytokine production were reduced (121). Mechanistically, neutralization of IL-12 activity at the time of HKL administration suppressed the enhancement of IFN-y and the reduction of IL-4 production, indicating that HKL-induced IL-12 production might be responsible for the observed adjuvant effect on cytokine production (121). Yeung and co-workers stated that *L.m.* infection elicited strong cell-mediated immune responses characterized by the presence of Ag-specific CD8 positive cytotoxic T cells. In line with this, *in vivo Listeria* application also rapidly activates innate immunity and induces high levels of IL-12, resulting in IFN-y production by NK cells and the induction of strongly Th1-polarized CD4 T cells (121).

Later on, in an asthma model the co-application of a single dose of HKL and the chicken eggwhite protein Ova proved sufficient to reverse an established airway hyperreactivity (AHR) in Ova-primed mice (45,122). As an adjuvant, HKL were shown to induce IFN-γ, IL-10, IL-12, and IL-18 secretion and to be efficient in converting Th2-dominated immune responses in protective immune responses in an allergen-specific manner. Moreover, using HKL in a food allergy model, Li and co-workers could demonstrate that the subcutaneous co-administration of modified peanut allergens and HKL was sufficient to protect against peanut-induced anaphylaxis in sensitized BALB/c mice, reduce bronchial constriction, plasma histamine levels, peanut-specific IgE levels, and symptom scores compared to sham-treated animals (55). Hereby, neither peanut allergens nor HKL provided alone had comparable protective effects. In spleenocyte cultures reduced levels of both IL-5 and IL-13, as well as increased IFN-y levels were observed only in mice that had been treated with modified Ara h 1–3 plus HKL (55). Therefore, Li and co-workers suggested a combination of modified peanut allergens and HKL to be more effective in conferring protection against peanut-induced anaphylaxis than the modified allergens alone. In another study application of HKL mixed with Ova in incomplete Freud's adjuvant (IFA) abrogated established Ova-specific Th2 responses, airway hyperreactivity (AHR) and airway inflammation in Ova-sensitized dogs

(121,123). This immune deviation was characterized by a rapid activation of innate immunity resulting in a significant decrease in allergen-specific IgE- and IL-4 levels as well as increased levels of Th1 associated markers such as allergen-specific IgG2a and IFN-y production. Furthermore, the therapeutic effect of HKL was associated with the production of IL-18. In accordance with the results presented by Yeung et al. (121), neutralization of IL-12 using a monoclonal antibody at the time of treatment with HKL and Ova abolished the induction of IFN-y and the reduction in IL-4, indicating that IL-12 also contributes to the observed effects (123). The protective effect of a single dose of HKL plus Ova in dogs lasted for at least 5 months, suggesting that administration of HKL plus antigen has antigen-specific long term effects on the immune system (123).

That HKL must be in close physical association with the allergen was elegantly shown by Hansen and co-workers (122). Ova-sensitized mice that received HKL thoroughly mixed with Ova together in the same footpad showed greatly reduced AHR upon Ova-challenge, whereas mice receiving HKL and Ova in different footpads displayed only minimal reductions in AHR (122). Taken together, these experiments showed that *Listeria* derived components may be clinically useful for the treatment of diseases caused by exaggerated allergen-specific Th2 responses, such as allergy and asthma.

1.4.2.2 HKL activate the innate immune system

Additional work on the immune modulating effects of heat killed *Listeria* showed that the protective effects of HKL-administration were mediated via the induction of innate immunity which resulted in strong Th1-dominated immune responses. Stock and co-workers used adoptive transfer experiments to show that the inhibitory effect of HKL was mediated by the induction of $CD8a^+CD11c^+DEC205^+$ DC producing IL-10 which induced CD4 positive regulatory T cells from naive $CD4^+CD25^+$ T cells (124). However, the underlying mechanisms of the beneficial effects remained unknown. Close-up studies revealed the immune modulating effects of HKL to be markedly reduced in $MyD88^{-/-}$ (myeloid differentiation factor 88) mice, which is a common adaptor protein for all bacterial Toll-like receptors (TLR), suggesting TLR-signalling to be involved (125).

L. monocytogenes contains several candidate TLR-ligands that might be responsible for the observed effects (Figure 3): lipoproteins, lipoteichoic acids, and peptidoglycanes which all bind to TLR2 (126), the bacterial DNA with its CpG-motifs that bind to TLR9 (127), and the TLR5-ligand flagellin (128,129), which is the major component of the bacterial flagellum. Further studies could show, that the immune modulating effects of HKL could still be observed in TLR9 knock out mice as well as in C3H/HeJ mice, which have a mutated TLR4

(55,123), leaving TLR5 and TLR2-ligands as potential causative agents for the observed effects.

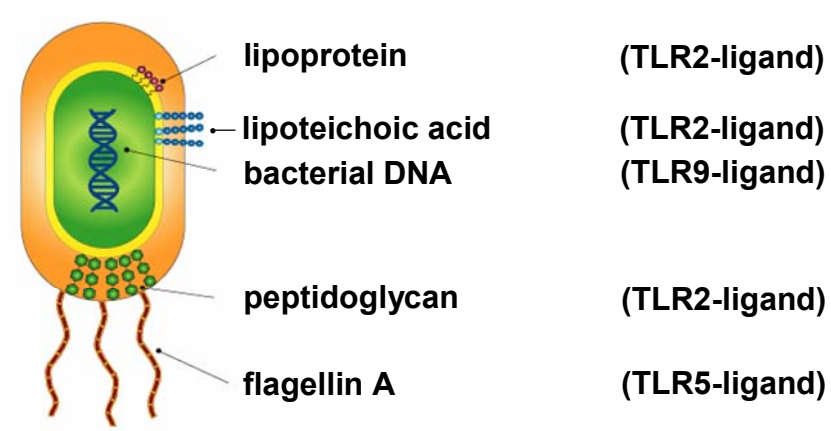


Figure 3: TLR-ligands in *L. monocytogenes.* For detailed information see text (Source: http://www.invivogen.com/popup .htm?images/bacteria_web.gif**).**

1.4.3 TLR-ligands

1.4.3.1 TLR-ligands are promising tools to modulate allergic immune responses

Single, defined microbial components such as TLR-ligands were evaluated as components of new therapeutic agents, since they should retain the potentially positive effects associated with the application of whole bacteria while minimizing risks and side effects. Diphtheria, and cholera toxin (130), as well as bacterial cell surface proteins (S-layer protein) (131) were tested.

A recombinant fusion protein constisting of Bet v 1 and the bacterial cell surface (S-layer) protein of *Geobacillus stearothermophilus* (rSbsC-Bet v 1) was shown to combine reduced allergenicity with immunomodulatory capacity. Although rSbsC-Bet v 1 contained all relevant Bet v 1-specific B and T cell epitopes, it did not trigger comparable histamine release to Bet v 1 alone, but induced IFN-γ and IL-10 production from both Bet v 1-specific Th2 cell clones and cells obtained from birch pollen allergic patients (131). Interestingly, no Th2-like responses were observed upon stimulation with rSbsC-Bet v 1 (131).

Among the different microbial components, pathogen associated molecular patterns (PAMP) are of special interest because of their potential to activate the host immune system and by this to influence or redirect existing immune responses. Toll like receptors (TLR) recognize pathogen associated molecular patterns (PAMPs) derived from bacteria and viruses and play an important role in the elimination of these pathogens. Importantly, engagement of TLRs

can stimulate antigen-presenting cells (e.g. DC and macrophages) to produce cytokines that favor Th1-type or T regulatory type (T_{reg})-immune responses. This suggests that TLR-ligands could be used as prophylactic and/or therapeutic adjuvants for the treatment of allergic diseases. Moreover, the usage of highly immunogenic compounds such as TLR-ligands is likely to abolish the need for additional adjuvants (132).

In line with this, monophosphoryl lipid A (MPL®) a nontoxic derivate of LPS, which is a TLR4-ligand from *Salmonella minnesota* (133) and CpG-containing oligodeoxynucleotides (ISS-ODN) activating TLR9, have been shown to induce Th1-cytokines and inhibit IgE synthesis in allergic patients (134,135). Consequently, both MPL® (133) and ISS-ODN (136) have been evaluated in clinical studies for their therapeutic potential in the treatment of allergic diseases where they showed improved symptom medication scores, reduced seasonal increases in IgE, and increased IgG1 and IgG4 levels, all promising results for the treatment of type I allergies (115,136-139). However, work on immune stimulatory ISS-ODN for the treatment of allergies was not continued probably due to low treatment efficacy.

1.4.3.2 TLR5-ligand flagellin is an interesting adjuvant candidate for allergen specific immunotherapy

One more candidate molecule for the development of new therapeutic vaccines is the TLR5-ligand flagellin. Flagellin is the main constituent of motility conferring flagella of gram positive and negative bacteria (128,140) and currently gained attention as adjuvant for prophylactic and/or therapeutic immune modulation in different diseases. Flagellin has various advantages as an immuno-adjuvant such as: (i) immunestimulatory properties at very low doses (picomolar range), (ii) enhancing effect on antigen-specific IgG and IgA production upon co-administration with the antigen, (iii) low toxicity, and (iv) its proteinous nature allowing the easy and reproducible generation of flagellin:antigen fusion proteins by recombinant DNA techniques (140-145).

The bacterial flagellum is a multiprotein complex, consisting of a basal body that anchors the structure in the inner and outer cell membrane, a flexible hook, and the flagellar filament consisting of more than 20000 repetitive flagellin molecules (146,147). While more than 50 genes are known to be involved in the regulated expression and function of the bacterial flagellum, the flagellar filament of most bacteria including *L. monocytogenes* is build up by only one major subunit, encoded by the 30.6 kDa flagellin flaA (147-149). Hereby, the long flagellum acts as a propeller confering bacterial mobility (118). Both the flagellin amino- and carboxy-termini are well conserved among different bacterial species, while the central portion shows a greater degree of variability in both length and amino acid composition, therefore being termed as “hypervariable region” (141,147,148,150). The protein filament of

each single flagellin molecule is folded back upon itself in a way that the amino- and carboxy-termini are physically located in close proximity to each other (151,152). Together both termini build three α-helices forming the central axis of the flagellum, whereas the species-specific hypervariable region forms the outer surface (152).
It was hypothesized that sequences within the N- or C-terminus or both may regulate flagellin export, flagellar assembly, and pro-inflammatory activity of flagellin, while the hypervariable domain is not involved in pro-inflammatory cell activation. Although, the hypervariable domain may be responsible for antigenic variation of the flagellar filament and therefore be a major antigenic determinant (141,147,150). In accordance with these assumptions, deletion of amino acids 95–108 in the N terminus and amino acids 441–449 in the C terminus completely abolished the pro-inflammatory activity of *Salmonella* flagellin C (150), indicating that N- and C-terminal regions together are recognized by TLR5.

1.4.3.3 Flagellin based vaccines

That flagellin might be a powerful tool to influence immune responses was first investigated in the 1990´s. These experiments were performed using flagellar display, a technique in which foreign peptides or proteins are introduced by genetic fusion into a surface exposed, dispensable region of flagellin. This leads to the efficient display of the introduced antigen on the surface of the highly repetitive flagellum, consisting of more than 20000 flagellin molecules and thereby acting as a multivalent antigen expressing multiple copies of the heterologous epitope (153,154). This display of foreign epitopes in the context of the bacterial flagellum causes the efficient production of antibodies directed against the inserted epitope (155). Moreover, in vaccine development usage of small molecules such as peptides may result in low immunogenicity, compared to proteins consisting of multiple epitopes or whole pathogens (156). The immunogenicity of peptides can be augmented by the usage of macromolecular carriers to which the desired epitope is either complexed or as in the case of engineered flagella covalently attached (156).
Using this technique flagellin from *Salmonella typhi* was successfully used in whole live vaccines as carrier molecule for displaying peptide based influenza vaccines at the bacterial cell surface (157). Administration of such live vaccines genetically engineered to express conserved influenza epitopes on their flagella was sufficient to confer protection against lethal influenza challenge (158). Interestingly, this protective effect was not influenced by preimmunization with flagellin alone (158). Additionally, intranasal immunization with a mixture of *Salmonella* flagella including conserved influenza epitopes, isolated from engineered bacteria, without additional adjuvant activated both cellular and humoral immunity. This treatment resulted in specific antibody responses that were sufficient to

protect against lethal virus challenge (153). In line with these studies, experiments comparing recombinant *Lactobacillus casei* expressing a *Salmonella typhimurium* flagellin C (fliC) fusion protein with the pathogenicity island 1 effector protein protein SipC of *Salmonella enterica* on the cell surface with SipC protein simply mixed with *L. casei* expressing fliC only showed that the fusion construct was more prone to induce Th1 responses in C3H/HeJ mice while the mixture of both components induced mixed Th2 and Th1 responses (159).
In parallel to the results obtained in this thesis initial results were reported from studies using flagellin containing fusion proteins for the treatment of infectious diseases (see discussion). However, up to now flagellin fusion proteins have not been investigated for their protential to treat allergic diseases.

1.4.3.4 TLR5 is expressed on many different cell types

Bacterial flagellins (including flaA derived from *L. monocytogenes*) were identified as the ligands for TLR5 (128,129). In accordance with their ability to activate TLR5, flagellin molecules derived from various bacteria, both gram positive and negative were shown to be pro-inflammatory at the picomolar range and to have strong immune modulatory activites (140,141,143). Hereby, different flagellin molecules induce inflammation via TLR5-mediated activation of immune cells (128) and are known to be virulence factors for pathogenic bacteria such as: *Vibrio cholerae, Campylobacter jejuni, Salmonella typhi* and *Legionella pneumophila* (118,143,148,160).
TLR5 is widely expressed in immune cells such as monocytes, macrophages, immature myeloid DC, both human and murine splenic DC, $CD11c^+$ intestinal lamina propria DC, and neutrophils, allowing the activation of these cells upon encountering flagellated bacteria (128,161-166). Moreover, human $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ T helper cells were shown to express TLR5 at levels comparable to those on monocytes and dendritic cells (167). In $CD4^+CD25^-$ effector T cells costimulation with anti-CD3 and flagellin induced enhanced proliferation and IL-2 production, whereas in human $CD4^+CD25^+$ T_{reg} cells flagellin treatment enhanced Fox p 3 expression and suppressive capacity (167,168). In accordance with these results murine $CD4^+CD25^+$ T_{reg} cells were also shown to express high levels of TLR1, 2, 4, 5, 7, and 8 mRNA, and stimulation with TLR4-ligand LPS was shown to enhance their suppressive capacity (167).
Moreover, intestinal epithelial cells were repeatedly shown to express TLR5 and flagellin can be considered a dominant innate immune activator of intestinal epithelial cells because flagellin stimulation is both necessary and sufficient to induce proinflammatory gene expression in these cells (169,170). These responses to flagellin are of special interest since the same epithelial cells show only a minimal responsiveness towards other TLR-ligands

such as LPS and CpG-DNA (171-173). On epithelial cells TLR5 expression is limited to the basolateral surface and is excluded from the luminal surface, thereby limiting inflammatory responses to endogenous gut bacteria to situations in which flagellated bacteria were able to break the intestinal epithelial cell barrier (129,141,143).

Therefore, both hematopoetic and non-hematopoetic cells contribute to flagellin-induced acute cytokine secretion, up-regulation of co-stimulatory molecules on DC, and flagellins ability to elicit IgG to itself and co-administered antigens (171). In this context the contribution of non-hematpoetic cells to humoral immunity might be to promote cell trafficking by secreting various chemokines in order to allow interaction between innate immune cells and lymphocytes (171).

1.4.3.5 Flagellin has potent adjuvant activities

Therefore, after the discovery of flagellins as TLR5-ligands, their potential use as adjuvants for infectious diseases and immunotherapy of allergic diseases was investigated. In 2007, Lee and co-workers treated BALB/c mice sensitized to the chicken egg white allergen Ova intranasally with a mixture of *V. vulnificus* flagellin B and Ova (140). Lee et al. could show that intranasal co-administration of *V. vulnificus* flagellin B and Ova significantly inhibited subsequent Ova-induced airway hyperreactivity, airway inflammation, and Th2-cytokine production in this mouse model of allergic asthma. Treatment with flagellin alone had no significant effect on airway responsiveness (140). Interestingly, the intranasal co-administration of flagellin B plus Ova did not induce Ova-specific production of the Th1-cytokine IFN-y from splenocyte cultures, suggesting the inhibitory effect of flagellin on Th2-mediated allergic responses to be rather caused by inhibition of Th2-responses without affecting Th1-responses (140). However, detailed mechanisms of the inhibitory effect of flagellin on Th2-mediated allergic responses are still unknown.

2. Aim

2.1 Working hypothesis

Previous studies demonstrated bacterial components to modulate allergic Th2 responses, which was not observed in TLR9 and TLR4 deficient mice. In parallel, as an adjuvant TLR5-ligand flagellin was shown to induce specific immune responses. Hereby, flagellin induces a Th1-biased activation of TLR5 expressing antigen presenting cells, making it an interesting tool for allergen specific immunotherapy. If this Th1-biased activation takes place in the presence of an allergen, the resulting immune response towards the allergen may be shifted from a predominant Th2- towards a Th1- or regulatory T cell response. This effect may further be enhanced if both, adjuvant (flagellin) and antigen are administered as a covalent fusion protein. Here, proximity of the TLR-ligand to an antigen is hypothesized to promote the adjuvant effect by simultaneously targeting and activating the same effector cell.

2.2 Working program

The overall aim was to test whether TLR5-ligand flagellin or fusion proteins containing flagellin might be used as vaccines for the treatment of type I allergies. Therefore, flagellin and fusion proteins consisting of flagellin and different allergens should be generated and assessed for their immune modulating properties *in vitro* and *in vivo*. Since heat killed *Listeria* were repeatedly shown to have strong immune modulating abilities and to induce protective Th1 immune responses in a TLR-dependent manner, flagellin A derived from *Listeria monocytogenes* was selected as model flagellin in this thesis. As fusion partners, chicken Ovalbumin (Ova), major peach (Pru p 3), and peanut (Ara h 2) allergens were chosen. All proteins should be cloned, expressed using the *E. coli* expression system, and purified via chromatographic methods. The quality of the purified proteins had to be assessed in respect to protein purity, formation of secondary structure elements, endotoxin content, and the proteins' ability to activate human and murine TLR5. The different constructs were to be tested for their properties to modulate innate and adaptive immune responses. For subsequent immunological studies it was decided to use the fusion protein of flaA and ovalbumin (rflaA:Ova) as model protein in a proof of concept study to investigate potential immune modulation and its potential mechanism. Due to a lack of comparable *in vitro* assays, fusion proteins including clinical relevant food allergens rflaA:Pru p 3 and rflaA:Ara h 2 could only be tested for their potency to induce DC activation. Therefore, *in vitro* differentiated murine DC-subsets (mDC and pDC) were either stimulated alone or in co-cultures with Ova-TCR-transgenic CD4 T cells and evaluated for cell activation and cytokine

profile. To characterize the mechanism of potential immune modulation by rflaA:Ova, neutralizing antibodies, inhibitors of endocytosis, and cells derived from TLR-signaling deficient knock out mice (e.g. MyD88$^{-/-}$) were applied. Finally, in order to support and substantiate the results obtained *in vitro,* rflaA and the rflaA:Ova fusion protein had to be tested for their therapeutic and prophylactic potency in a murine model of Ova-induced intestinal allergy.

The results obtained in this study should provide evidence whether flagellin and fusion proteins containing flagellin might be used as vaccine candidates for the treatment of severe type I allergies.

3. Material and methods

3.1 Cloning, expression and purification of flagellin, allergens and flagellin:allergen fusion proteins

3.1.1 Cloning of flagellin A, rOva, and rflaA-Ova fusion protein

A synthetic gene encoding for ***Listeria monocytogenes*** **flaA (Acc.no. X65624.1,** Geneart, Regensburg) served as template for the cloning. The flaA cDNA was elongated via PCR adding the NdeI restriction side at the N-terminus and the XhoI sequence on the C-terminus (flaA: for: 5´-catatgaaagtaaatactaatatcattagcttgaaaacacaagaatatc-3´, rev: 5´-ctcgagt tagctgttaattaattgag-3´, template amount: 1 ng per reaction, annealing temperature: 41 to 51°C for 30 seconds, elongation temperature: 72°C for 1 minute, 25 PCR-cycles, polymerase: 1 U Platinum Taq polymerase (Invitrogen, Darmstadt)). The resulting PCR-product was cloned into pCR®4-TOPO (TOPO Cloning Kit for Sequencing (Invitrogen) according to the manufacturers recommendations, and transformed into *E. coli* TOP10 cells (Invitrogen). Purified pCR®4-TOPO plasmids were digested with NdeI and XhoI, both 20 U per reaction for 1 h at 37°C (NEB, Frankfurt). Subsequently, restriction enzymes were heat-inactivated (80°C for 20 minutes), the insert was purified via gel extraction (Qiagen, Hilden), and ligated (approximately 5 to 15 ng DNA, Quick Ligation Kit, NEB) into the likewise digested and purified pET15b plasmid (1 ng per reaction, Novagen, Darmstadt, for vector chard see Repository figure 17). This resulted in an open reading frame coding for a N-terminal His_6-Tag, a thrombin protease restriction side and the flaA protein with a calculated molecular weight of 30.5 kDa. Sequence identity was verified by analytical restriction digest (NdeI plus XhoI, both 20 U per reaction for 1 h at 37°C, NEB) and bidirectional Sanger sequencing (Eurofins, MWG, Operon, Ebersberg).

The cDNA of **chicken ovalbumin (Ova, Acc.no. NM_205152)** was elongated by NdeI (N-terminus) and XhoI (C-terminus) restriction sites (Ova: for: 5´-tatatatacatatgatgggctcc atcggcgc-3´, rev: 5´-tatatatactcgagtcattaaggggaaacacatctgcc-3´ template amount: 1 ng per reaction, annealing temperature: 40°C for 30 seconds, elongation temperature: 72°C for 1.5 minutes, 25 PCR-cycles, polymerase: 1 U Platinum Taq polymerase (Invitrogen)) using an Ova-containing plasmid (kindly provided by Prof. G. Sutter) as template, and cloned into pET15b (Novagen) (for technical details see above). This resulted in an open reading frame encoding for an N-terminal His_6-Tag, a thrombin protease cutting side and the rOva protein with an overall molecular weight of 44 kDa (for vector chard see Repository figure 17). Sequence identity was verified via analytical restriction digest (NdeI plus XhoI, both 20 U per

reaction for 15 minutes at 37°C, NEB) and bidirectional Sanger sequencing (Eurofins, MWG, Operon).

The **rflaA:Ova fusion protein** was generated by assembly PCR using flaA cDNA (Acc.no. X65624.1, Geneart) elongated with a NdeI restriction site at the 5`-end and a sequence stretch complemenary to 5´-end of Ova at its 3´-end (flaA:Ova (flaA extend): for: 5´-catatgaaagtaaatactaatatcattagcttgaaaacacaagaatatc-3´, rev: 5´-gctgcgccgatggagcccatg ctgttaattaattgagtt-3´, template amount: 1 ng per reaction, annealing temperature: 40°C for 30 seconds, elongation temperature: 72°C for 1 minute, 25 PCR-cycles, polymerase: 1 U Platinum Taq polymerase (Invitrogen)). The Ova cDNA (Acc.no. NM_205152) was elongated with a XhoI restriction site at the 3´-end and a sequence stretch complementary to the 3´-end of flaA at the 5´-end (flaA:Ova (Ova extend): for: 5´-aactcaattaattaacagcatgggct ccatcggcgcagc-3´, rev: 5´-ctcgagtcattaaggggaaacacatctgcc-3´, template amount: 1 ng per reaction, annealing temperature: 40°C for 30 seconds, elongation temperature: 72°C for 1 minute, 25 PCR-cycles, polymerase: 1 U Platinum Taq polymerase (Invitrogen). Using the overlapping regions the two elongated PCR-products were fused in an assembly PCR (flaA:Ova (fusion): for: 5´-catatgaaagtaaatactaatatcattagcttgaaaacacaagaatatc-3´, rev: 5´-ctcgagtcattaaggggaaacacatctgcc-3´, annealing temperature: 55°C for 30 seconds, 25 PCR-cycles, elongation temperature: 72°C for 2 minutes, polymerase: 1 U Platinum taq, Invitrogen), using 1 µl of the 1 to 10 diluted and unpurified PCR-products of the initial PCR-reactions as templates. For cloning of the fusion product into pGEM®T easy (Promega, Mannheim) PCR-products were A-tailed. In order to remove salts PCR reactions were first gel extracted using the gel extraction kit (Quiagen) according to the manufacturers' recommendations. For A-tailing 1 µl of 25 mM $MgCl_2$, 2 µl 2 mM dATP (both Fermentas, St. Leon-Rot), 5 µl of 10x PCR-buffer, and 0.4 U of Taq polymerase (Erlangen, kindly provided by Karin Metzner), were added to 5 µl of the gel extracted PCR-product and incubated at 72°C for 30 minutes in a total volume of 50 µl. A-tailed PCR-products were purified by standard ethanol precipitation and DNA was dissolved in 10 µl sterile water. For ligation into pGEM®T easy (Promega, according to the manufacturers recommendations) 3 to 4 µl of the A-tailed PCR-products were used. Ligated pGEM®T easy plasmids with the fusion sequence were transformed into *E. coli* TOP10 cells. To identify positive clones over night cultures were inoculated and colony-PCR was performed (template: 1 µl of overnight culture, colony PCR: for: 5´-attatgctgagtgatatcccgc-3´, 5´-taagatatcacagtggattta-3´, annealing temperature: 50°C for 30 seconds, elongation at 72°C for 2 minutes, 40 cycles, 0.4 U Taq polymerase (Erlangen), 5 µl of 10x PCR-buffer, 0.4 µl of 10 mM dNTP-mix, 1.2 µl of 25 mM MgCl2, 1 µl of 10 pmol primers in a total volume of 20 µl). Colony-PCR reactions were analyzed by agarose gel electrophoresis and positive clones were digested with NdeI and XhoI (both 20 U per reaction for 1 h at 37°C, NEB), restriction enzymes were heat-inactivated (80°C for 20

minutes), and the PCR product, encoding for a protein with a molecular weight of 74 kDa was purified and ligated into the likewise digested and purified pET15b as described above (for vector chard see Repository figure 17). Sequence identity was verified via analytical restriction digest (NdeI plus XhoI, both 20 U per reaction for 1 h at 37°C, NEB) and bidirectional Sanger sequencing (Eurofins, MWG, Operon).

3.1.2 Expression and purification of flagellin A, rOva, and rflaA-Ova fusion protein

For protein **expression** 1.8 litres of standard LB-medium plus 0.5 ml antifoam B emulsion (Sigma, Steinheim) and 50 mg/l carbenicillin (Roth, Karlsruhe) in five litre shaking flaskes with baffles were inoculated with approximately 100-125 ml over night culture of *E. coli* BL21 Star DE3 cells (Invitrogen) transformed with the respective pET15b constructs. Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.75 to 1 mM, Fermentas) at OD_{600} = 0.5. After 5 to 6.5 hours of induction (37°C and 220 rpm) cells were harvested by centrifugation (7.500 g for 10 min), cell pellets were immediately shock frozen with liquid nitrogen (rflaA and rOva) or subjected to N_2 after resuspension in 50 ml 20 mM Tris (pH 8.5, plus 0.2 M NaCl supplemented with protease inhibitors, Roche, Grenzach-Whylen) (rflaA:Ova). All samples were stored at -80°C until purification.

For further **protein isolation**, cells were taken up in 100 ml 50 mM phosphate buffer (pH 8.0, supplemented with 0.5 M NaCl, 2 mM EDTA (Titriplex III; Merck, Darmstadt)) and 0.5 mg/ml lysozyme (Boehringer, Mannheim) (rflaA and rOva), or 50 ml 20 mM Tris (pH 8.5, supplemented with 0.2 M NaCl, and 1 tablet protease inhibitor (Roche) (rflaA:Ova), and incubated for 15 minutes at room temperature. Cell suspensions were treated with 0.2 to 0.4% Triton X-100 (Sigma), and 2 U benzonase/ml (Novagen) for 30 minutes at room temperature. $MgCl_2$ (4 mM) was added to rflaA and rflaA:Ova preparations. Further cell lysis was performed by sonication (4 times 15 sec in 2 min with 100 W power on ice) and repeated freezing and thawing in N_2. Insoluble constituents were separated via centrifugation at 10.000 g for 10 min at 4°C. Inclusion bodies from pellets were washed twice with 150 ml 50 mM phosphate buffer (pH 8.0) plus 0.5 M NaCl and solubilized (1 h incubation on ice) with 200 ml 50 mM phosphate buffer (pH 8.0) supplemented with 0.5 M NaCl and 6 M urea (flaA), or washed twice with 120 ml of 20 mM Tris (pH 7.5) plus 10 mM EDTA, and 1% Triton X-100 (rflaA:Ova). The **solubilization** of rflaA:Ova was performed (1 h incubation on ice) with 125 ml CAPS (20 mm, pH 11.0) supplemented with 0.3% sarcosyl-lauryl-sulfate (SLS) (Novagen) and 1 mM dithiothreitol (DTT, Molecula, UK). For refolding of rflaA:Ova the Protein Refolding Kit (Novagen) was used according to the manufacturers recommendations. Insoluble constituents were removed via centrifugation at 27.000 g for 20 min. Recombinant Ova was

prepared from cell lysates under native conditions. Solubilized proteins were subjected to filtration (0.2 µm nitro cellulose filter, Merck).

Purification of proteins was performed by a two-step chromatography strategy: immobilized metal ion affinity chromatography (IMAC) followed by size exclusion chromatography (SEC). After addition of 20 mM imidazol the clarified cell lysates were applied either on a XK-16 column (Amersham, Munich) loaded with 18 ml nickel nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) (rflaA) or on a 5 ml HisTrap FF crude column (GE healthcare, Munich) (rOva and rflaA:Ova). After washing with 5 column volumes of 50 mM phosphate buffer (pH 8.0, 0.5 M NaCl, 20 mM imidazol) the proteins were eluted by 50 mM phosphate buffer (pH 8.0, 0.5 M NaCl) supplemented with 100 mM imidazol (rOva) or by a linear gradient of 0 to 500 mM imidazol (rflaA:Ova). Recombinant flaA was refolded on the column with a linear gradient from 6 M to 0 M urea (in 90 min with a flow rate of 3 ml/min) and subsequently eluted with a stepwise gradient with 150, 300 and 500 mM imidazol in a 50 mM phosphate buffer (pH 8.0, 0.5 M NaCl). Fractions containing the target protein were checked by SDS-PAGE, pooled and filtered through a 0.2 µm syringe filter (Minisart, Switzerland). In order to improve protein purity and remove endotoxins a size exclusion chromatography using a HiLoad™ XK-16/60 Superdex™ 200 prep grade (Amersham) was performed. Running buffers were 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS, pH 7.4, 0.5 M NaCl) (rflaA and rOva), and phosphate buffered saline (PBS, pH 7.1 with 350 mM NaCl and 0.04 % SLS, Novagen) (rflaA:Ova). Purified proteins were either stored in PBS at -20°C (rOva), at -20°C mixed with 50% glycerol (rflaA), or at 4°C (rflaA:Ova).

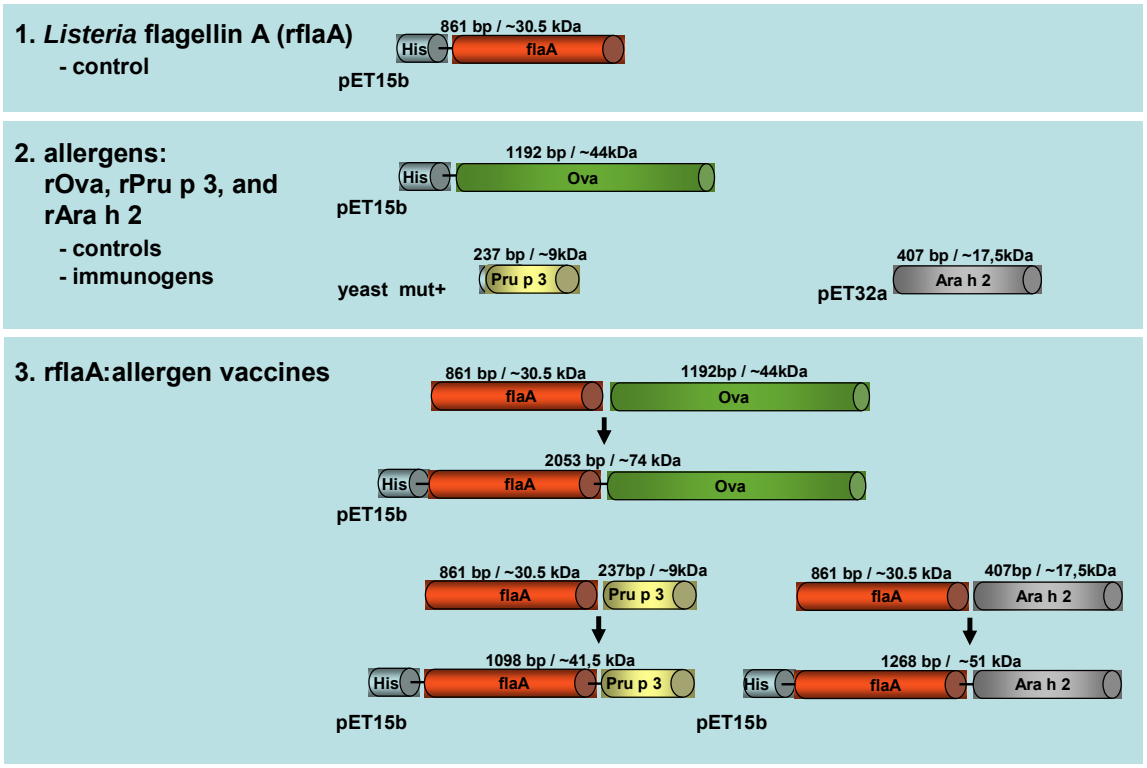


Figure 4: Preparation of *Listeria monocytogenes* flagellin A, control allergens and chimeric flagellin:allergen constructs. For detailed information see text.

3.1.3 cDNA-cloning of rflaA:Pru p 3 and rflaA:Ara h 2 fusion proteins

The **rflaA:Pru p 3 fusion protein** was generated by assembly PCR using flaA cDNA (Acc.no. X65624.1, Geneart) elongated with a NdeI restriction site at the 5`-end and a sequence stretch complemenary to 5´-end of Pru p 3 at its 3´-end (flaA:Pru p 3 (flaA extend): for: 5´-catatgaaagtaaatactaatatcattagc-3´, rev: 5´-gcaaatgttaactcaattaattaacagcataacatgtg gccaagtg tccagc-3´, template amount: 1 ng per reaction, annealing temperature: 41 to 56°C for 30 seconds, elongation temperature: 72°C for 1 minute, 25 PCR-cycles, polymerase: 1 U Platinum Taq polymerase, Invitrogen). The Pru p 3 cDNA (Acc.no. AY792996) was elongated with a XhoI restriction site at the 3´-end and a sequence stretch complementary to the 3´-end of flaA at the 5´-end (flaA:Pru p 3 (Pru p 3 extend): for: 5´-gcaaa tgttaactcaattaattaacagcataacatgtggccaagtgtccagc-3´, rev: 5´-ccaccaactgcgccaccgtgaagt gactcgag-3´, template amount: 1 ng per reaction, annealing temperature: 41 to 56°C for 30 seconds, elongation temperature: 72°C for 1 minute, 25 PCR-cycles, polymerase: 1 U Platinum Taq polymerase, Invitrogen). Using the overlapping regions the two elongated PCR-products were fused in an assembly PCR (flaA:Pru p 3 (fusion): for: 5´-aaagtaaatactaatatcattagc-3´, rev: 5´-ccaccaactgcgccaccgtgaagtgactcgaga-3´, annealing temperature: 63°C for 30 seconds, 25 PCR-cycles, elongation temperature: 72°C for 2

minutes, polymerase: 1 U Platinum Taq polymerase (Invitrogen), using 1 µl of the 1 to 10 diluted (non-purified) PCR-products of the initial PCR-reactions as templates. The resulting PCR product was ligated into the pCR®4-TOPO vector using the TOPO Cloning Kit for Sequencing (Invitrogen) and amplified in *E. coli* TOP10 cells (Invitrogen). Plasmids containing the fusion product were purified via the QIAprep Spin Miniprep Kit (Qiagen) and digested with NdeI and XhoI (both 20 U per reaction for 1 h at 37°C, NEB). The fusion product was purified via gelextraction (Gel Extraction Kit, Qiagen) and ligated (using 1 µl of the purified construct containing approximately 5 to 15 ng of DNA) into the likewise digested and purified pET15b vector (Novagen, for vector chard see Repository figure 17) using the Quick ligation Kit (NEB). This resulted in an open reading frame consisting on protein level of a His_6-Tag, a thrombin protease cutting side and the rflaA:Pru p 3 fusion protein with an overall size of 41.5 kDa. The pET15b_rflaA:Prup3 construct was transformed into *E. coli* BL21 Star DE3 (Invitrogen) expression cells. Sequence identity was verified via analytical restriction digest (NdeI plus XhoI, both 20 U per reaction for 1 h at 37°C, NEB) and bidirectional Sanger sequencing (Eurofins, MWG Operon).

The **rflaA:Ara h 2 fusion protein** was generated by assembly PCR using flaA cDNA (Acc.no. X65624.1, Geneart) elongated with a NdeI restriction site at the 5`-end and a sequence stretch complemenary to 5´-end of Ara h 2 at its 3´-end (flaA:Ara h 2 (flaA extend): for: 5´-tatatacatatgaaagtaaatactaatatcattagcttgaaaacacaagaatatc-3´ rev: 5'-cctgcagttcccactg ctggcggctgttaattaattgagtt-3', template amount: 1 ng per reaction, annealing temperature: 40°C for 30 seconds, elongation temperature: 72°C for 1 minute, 25 PCR-cycles, polymerase: 1 U Platinum Taq polymerase, Invitrogen). The Ara h 2 cDNA (Acc.no. L77197) without the sequence encoding for the N-terminal signal peptide (GCTCACCATACTAGTAGCCCTCGCCCTTTTCCTCCTCGCTGCCCACGCATCTGCG) was elongated adding a XhoI restriction site to the 3´-end and a sequence stretch complementary to the 3´-end of flaA to the 5´-end (flaA:Ara h 2 (Ara h 2 extend): for: 5'-aactcaattaatt aacagccgccagcagtgggaactgcagg-3', rev: 5'-ctcgagtcaatagcgatcgcggccgctatata-3', template amount: 1 ng per reaction, annealing temperature: 40°C for 30 seconds, elongation temperature: 72°C for 1 minute, 25 PCR-cycles, polymerase: 1 U Platinum Taq polymerase, Invitrogen). A pET32a plasmid containing the Ara h 2 cDNA kindly provided by Prof. P. Rösch (University of Bayreuth) served as template. Furthermore, TATATA-sequences were added to the 5'-end of rflaA- and the 3'-end of the Ara h 2-sequence in order to allow direct cleavage of the PCR product. Using the overlapping regions the two elongated PCR-products were fused in an assembly PCR (flaA:Ara h 2 (fusion): for: 5´-tatatacat atgaaagtaaatactaatatcattagcttgaaaacacaagaatatc-3´, rev: 5'-ctcgagtcaatagcgatcgcggccgctat ata-3', annealing temperature: 48°C for 30 seconds, 25 PCR-cycles, elongation temperature: 72°C for 1.5 minutes, polymerase: 1 U Platinum Taq (Invitrogen), using the 1 to 20 diluted

(non-purified) PCR-products of the initial PCR-reactions as templates. The fusion construct was cleaned by extraction from a 1.5% agarose gel (gel extraction kit, Qiagen). The purified fusion product was digested with NdeI and XhoI (NEB), and restriction enzymes were removed via PCR Purification Kit (Qiagen). Afterwards the fusion product was ligated (Quick ligation Kit according to the manufactureres recommendations, NEB), into the likewise digested and purified pET15b plasmid (1 ng per reaction, Novagen, for vector chard see Repository figure 17). This resulted in an open reading frame consisting on protein level of a His_6-Tag, a thrombin protease cutting side and the rflaA:Ara h 2 fusion protein, with an overall molecular weight of 51 kDa. The pET15b_rflaA:Ara h 2 was transformed into *E. coli* BL21 Star DE3 expression cells (Invitrogen). Sequence identity was verified via analytical restriction digest (NdeI plus XhoI, both 20 U per reaction for 1 h at 37°C, NEB) and bidirectional Sanger sequencing (Eurofins, MWG, Operon).

3.1.4 Expression and purification of recombinant allergens and fusion proteins

Recombinant **Ara h 2** was prepared by Sonja Wolfheimer and Stefan Mende using a pET32a_Ara h 2 construct kindly provided by Prof. P. Rösch (University of Bayreuth), and *E. coli* Origami cells (Novagen) according to the protocol published by Lehmann et al. (38). Recombinant **Pru p 3** was prepared by Sven Pokoj using *P. pastoris* expression cells according to the protocol described elsewhere (174).

For expression of the **rflaA:Pru p 3 and rflaA:Ara h 2** fusion proteins two times 1.8 litres of standard LB-medium plus 1 ml antifoam B emulsion (Sigma) and 50 mg/l carbenicillin (Roth) in 2 litre benchtop laboratory fermenters with air bubbler, magnetic stir bar and temperature control (Schott, Mainz) were inoculated with 200 ml over night culture each. The culture was incubated at 37°C and 700 rpm. At OD_{600} = 0.5 the expression was induced using 0.75 mM IPTG (Fermentas). 4.5 hours (rflaA:Ara h 2) or 7 hours (rflaA:Pru p 3) post induction cells were harvested by centrifugation (7500 g for 10 min), cell pellets were shock-frozen with liquid nitrogen and stored at -80°C. Cell lysis and purification of rflaA:Pru p 3 and rflaA:Ara h 2 were performed according to the method described for rflaA (see above).

3.1.5 Determination of protein concentration

Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Solingen) according to the manufacturers recommendations. This methods combines the biuret reaction (reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium) with detection of Cu^{+} cations by bicinchoninic acid (BCA) for the colorimetric quantitation of total protein.

3.1.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Recombinant proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (175) (cross linker C = 5%, total bis/acrylamid 15%) under reducing (addition of 0.5 M DTT) or non-reducing conditions. Before electrophoresis, samples were mixed with sample buffer (6% SDS, 40% glycerine, 0.1% bromphenoleblue, 60 mM Tris, pH 8.0) and cooked for 5 minutes at 95°C. Protein separation was performed at 150 V (running buffer 25mM Tris/HCl, 250 mM Glycin, 0.1% SDS, pH 8.3) using MiniProtein III apperatures (BioRad, Munich).

3.1.7 Reduction and alkylation

For reduction and alkylation 10 ml rflaA:Ova, containing approximately 30 mg of protein were taken up in 30 ml 200 mM Tris-HCl, 8 M urea (Merck, Darmstadt) and 450 mM DTT (Molecula, UK) and incubated at 45°C for 1 h, subsequently 500 mM iodacetamide (Merck) was added and the reaction was incubated for 20 minutes at room temperature in the dark. Reduced and alkylated rflaA:Ova (rflaA:Ova R/A) was dialysed against PBS for approx. 3 to 4 days (Slide-A-Lyzer Dialysis Casette, 3500 MWCO, 12-30 ml capacity, Thermo Scientific, Dreieich, 4°C, against 5 l PBS, with a daily buffer change) .

3.1.8 Limulus amebocyte lysate test

Endotoxin concentration was determined via *Limulus* amebocyte lysate (LAL) test according to the manufactures recommendations using the automated PTS LAL device (PTS LAL, Charles River, Sulzfeld). Here, each cartridge contains LAL-reagents, chromogenic substrate, and a defined amount of endotoxin as standard. Endotoxin concentration within samples is determined by quantification of the chromogenic reaction using an internally provided standard.

3.1.9 Circular dichroism spectroscopy

Purified proteins were adjusted to a concentration of 0.022 to 0.027 nmol/ml (200 µg/ml) and extensively dialyzed against 10 mM phosphate buffer. Circular dichroism (CD) spectra were recorded using a JASCO J-810 spectropolarimeter (Jasco, Gross-Umstadt) with constant N_2 flushing at 20°C. Measurements were performed in a quartz glass cuvette (1 mm) with a step width of 1 nm and a band width of 1 nm. The spectral range was 180–260 nm at 50 nm/min. Ten scans were accumulated, spectra obtained with buffer were subtracted. The results were expressed as mean residue molar ellipticity $[H]_{MRD}$ and analyzed for folding of secondary structure elements. Hereby, correctly folded α-helical structures display minima at 208 and 220 nm as well as a typical w-shape of the spectrum.

3.2 *In vitro* assays

3.2.1 TLR5-activation assay

HEK293 cell lines stably transfected with the murine or human TLR5 (InvivoGen, France) were cultured (5% CO_2, 37°C, 95% humidity) in DMEM (Biochrome, Berlin) containing 10% FCS (Biochrome), L-glutamine (0.15 mg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml, both Invitrogen), and blasticidin (10 µg/ml, InvivoGen). Wild type HEK293 cells were cultured as indicated above without blasticidin. For TLR5-activation assays $4x10^4$ cells per well were seeded in 48-well plates (Nunc, Wiesbaden) and cultured in DMEM containing 2% FCS, L-glutamine, penicillin, and streptomycin over night. The cells were stimulated with equimolar amounts of rflaA, rOva, rPru p 3, rAra h 2, rflaA:Ova, rflaA:Ova R/A, rflaA:Pru p 3, or rflaA:Ara h 2 for 22 h. Supernatants were stored at -20°C, and concentration of human IL-8 in the supernatant was determined by ELISA (see 3.2.6).

3.2.2 Mice

BALB/c, C57BL/6, and DO11.10 (BALB/c) mice were purchased from Jackson laboratories (USA). OT-II (C57BL/6), MyD88$^{-/-}$ (C57BL/6), Trif$^{-/-}$ (C57BL/6), and MyD88$^{-/-}$Trif$^{-/-}$ (C57BL/6) (176) mice were provided by Dorothea Kreuz out of the animal facility of the Paul-Ehrlich-Institut. All animals were kept under specific pathogen free conditions at the animal facility of the Paul-Ehrlich-Institut. Mouse experimental work was carried out using 8- to 20-week old mice in compliance with regulations of german animal welfare (for more information see table 13).

3.2.3 In vitro generation of bone marrow derived murine dendritic cells

Bone marrow cells were isolated from femur and tibia of BALB/c, C57BL/6, MyD88$^{-/-}$ (C57BL/6), Trif$^{-/-}$ (C57BL/6), and MyD88$^{-/-}$Trif$^{-/-}$ (C57BL/6) (176) mice. After lysis of red blood cells using red blood cell lysis buffer (Sigma), bone marrow cells were seeded in RPMI 1640 (Gibco, Darmstadt) supplemented with 10% FCS (Biochrome), 1 mM sodium pyruvate (Sigma), 10 mM HEPES (Merck), penicillin (100 U/ml), streptomycin (100 µg/ml) (both Invitrogen), and 0.1 mM ß-mercapto-ethanol (Sigma). Differentiation of bone marrow cells into myeloid dendritic cells (mDC) and plasmacytoid DCs (pDC) was achieved by culturing the cells for 8 days in the presence of either 100 ng/ml GM-CSF (R&D Systems, Wiesbaden) or 100 ng/ml Flt-3L (R&D Systems), respectively. Cultures containing Flt-3L were cultivated

with one medium change at day 4. Medium change of GM-CSF cultures was performed every two days depending on the status of the culture.

3.2.4 In vitro stimulation of bone marrow derived murine dendritic cells

On day 8 of mDC and pDC culture loosely adherent cells were collected by pipetting and centrifugation (5 minutes at 226 g, RT), $3.2x10^5$ cells/ml were seeded in 24-well plates (Nunc), and stimulated with equimolar amounts of either rflaA (6.9 µg/ml), rOva (10 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), rflaA:Ova (16.9 µg/ml), rflaA:Ova (R/A) (16.9 µg/ml), and LPS (10 µg/ml, Sigma) for 24 or 72 h. The amounts of rflaA:Ova (16.9 µg/ml) were adjusted to an equimolar amount of rOva and rflaA. Supernatants were stored at -20°C, and cytokine levels in the supernatant of stimulated cells were determined by ELISA (see 3.2.6).

In order to investigate whether rflaA was able to induce cytokine production from mDC, BALB/c $3.2x10^5$ mDC/ml were stimulated in 24-well plates (Nunc) with equimolar amounts of rflaA and rflaA:Ova equivalent to 0.25 to 64 µg/ml flagellin for 24 h. To exclude influences of putative LPS contaminations on the observed effects, mDC were stimulated in parallel with LPS amounts ranging from 1 pg/ml to 10 µg/ml for 24 h. Supernatants were stored at -20°C and analyzed for IL-6 and IL-10 production by ELISA (see 3.2.6).

To determine the kinetics of cytokine secretion $3.2x10^5$ BALB/c mDC/ml were seeded in 24-well plates (Nunc), and stimulated with LPS (10 µg/ml) and equimolar amounts of rOva (10 µg/ml), rOva plus LPS (10 µg/ml each), rflaA (6.9 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), rflaA:Ova (16.9 µg/ml) and rflaA:Ova R/A (16.9 µg/ml). Supernatants were collected after 2, 4, 6, 8, 12, and 24 h, stored at -20°C, and checked for the production of IL-6 and IL-10 by ELISA (see 3.2.6).

For stimulation of mDC with different TLR-ligands TLR4-ligand LPS was purchased from Sigma (LPS from *salmonella enterica* serotype abortus equi, Sigma), TLR2-ligand $Pam_3CysteineK_4$, TLR2/6-ligand FSL-1, TLR3-ligand poly-I:C, TLR7/8-ligand R848 and TLR8-ligand *E. coli* RNA were purchased from InvivoGen (InvivoGen). In addition to TLR5-ligand flagellin (see 3.1.2) heat killed *Listeria monocytogenes* (InvivoGen) were applied. Modified Virus Ankara (MVA) was kindly provided by Christoph Bohnen (Paul-Ehrlich Institut, Langen). mDC were prepared from C57BL/6 bone marrow (see 3.2.3) and either stimulated for 24 h with the different TLR-ligands or infected for 2 h with MVA, washed, and incubated for another 22 h. Supernatants were collected, and analyzed for secretion of IL-1β, IL-6 and IL-10 by ELISA (see 3.2.6), cells were stained for CD11b, CD11c, B220, and the activation markers CD40 and CD69 (see 3.2.5).

3.2.5 Flow cytometry and intracellular cytokine staining

For assessment of expression levels of co-stimulatory molecules, MHC molecules and TLR5 on the cell surface, mDC and pDC were stimulated as indicated (see 2.2.4) and stained with FITC-conjugated anti-mouse CD40, CD80, CD86 or MHC class II mAbs (*e*Bioscience, USA), or PE-conjugated anti-mouse TLR5 (Abcam, USA), CD40, CD69, and MHC class I mAbs (*e*Bioscience). Additionally, cells were stained with Pacific Blue-conjugated anti-mouse CD11b (Invitrogen), allophycocyanin-conjugated anti-mouse CD11c (BD Biosciences, Heidelberg) and phycoerythrin-Cy5 conjugated anti-mouse B220 (BD Biosciences) mAbs to gate mDC and pDC populations. Appropriate isotype controls for all antibodies were performed. FITC or PE intensity of $CD11b^+CD11c^+B220^-$ cells (mDC), or $CD11b^-CD11c^+B220^+$ (pDC) was measured by flow cytometry, using a BD LSR II cytometer (BD Biosciences). Data were analysed using FlowJo V.7.2.4 (Treestar Inc., USA). For staining of cell surface markers approximately $6.4x10^5$ cells were stained in a volume of 50 µl FACS-buffer (see table 9) with the indicated amount of antibodies (see table 8) for 20 minutes at 4°C in the dark, washed with 1 ml FACS-buffer, taken up in 250 to 400 µl FACS-buffer, and fixed using 50 µl 4% paraformaldehyde (Sigma). For determination of DC purity mDC and pDC preparations were stained with FITC-conjugated anti-mouse CD3 and CD19 mAbs (both Southern Biotech, USA, see table 8). Stained cells were stored in the fridge until measurement.

For intracellular cytokine staining mDC or pDC were stimulated with the different constructs for 22 h and subsequently 4 h in the presence of 1 µl/ml Golgi Plug (BD Biosciences). Cells were harvested, washed in FACS-buffer and incubated with 1 µg Fc-block (CD16/CD32, Fcγ III/II receptor, BD Biosciences) for 15 minutes at 4°C. In order to gate for mDC and pDC populations, cells were stained for Pacific Blue-conjugated anti-mouse CD11b (Invitrogen), allophycocyanin-conjugated anti-mouse CD11c (BD Biosciences) and phycoerythrin-Cy5 conjugated anti-mouse B220 (BD Biosciences) mAbs. Subsequently, cells were fixed and permeabilized in 250 µl fixation/permeabilization solution (BD Biosciences) at 4°C in the dark over night. Cells were washed once using 1 ml perm/wash solution (BD Biosciences). Intercellular cytokines IL-6 and IL-10 were stained using PE-conjugated antibodies (both *e*Bioscience) in volume of 50 µl perm/wash solution. The staining was performed for 1.5 to 2 h at 4°C in the dark. Subsequently, cells were washed in perm/wash solution and taken und in 300 µl FACS-buffer. PE-intensity of $CD11b^+CD11c^+B220^-$ cells (mDC), or $CD11b^-CD11c^+B220^+$ (pDC) was measured by flow cytometry, using a BD LSR II cytometer (BD Bioscience). Data were analysed using FlowJo V.7.2.4 (Treestar Inc.).

For analysis of $CD4^+$ T cell purity $0.5x10^5$ total cells were stained using 5 µl FITC-conjugated anti mouse CD4 antibody (Miltenyi Biotech, Bergisch-Gladbach), according to the procedure described above. Used amounts for all antibodies are indicated in table 8.

3.2.6 Cytokine ELISAs

Cytokines levels in the culture supernatant were measured by ELISA. The levels of human IL-8, and murine cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, and IFN-γ were determined using the BD OptEIA™ ELISA Sets (BD Biosciences) according to the manufacturers' recommendations. The levels of murine IL-2 were measured using an anti-mouse IL-2 antibody (clone JES6-1A12) in combination with a biotin anti-mouse IL-2 antibody (clone JES6-5H4), both used at a concentration of 0.5 µg/ml (both Biolegend, The Netherlands). The levels of murine TGF-ß, IL-17A, and IL-23 were measured using Ready-SET-GO! ELISA kits (eBiosciences) following the manufacturers recommendations. Cytokine concentrations in supernatants were either measured undiluted (for determination of TGF-β, IL-1β, IL-4, IL-10, IL-12p70, IL-17, and IL-23), diluted 1:80 to 1:200 (IL-6 and IL-8), 1:5 to 1:35 (IFN-γ), and 1:2 to 1:4 (IL-2). For dilution PBS with 10% FCS (Biochrom) was used. Absorbance (A =450 nm) was analyzed using a SpectraMAX340PC (Molecular Devices, USA) and data were processed using Excel (Microsoft, USA).

3.2.7 Blocking of endocytosis

To investigate the influence of endocytosis on cytokine secretion, BALB/c mDC ($3.2x10^5$ mDC/ml) in 24-well plates (Nunc) were preincubated for one hour with the inhibitor of actin polymerization cytochalasin D (5 µg/ml) and the inhibitor of lysosmal acidification bafilomycin A1 (100 ng/ml) either provided separate or together (both Sigma), both dissolved in DSMO (Fluka, Neu-Ulm). Appropriate amounts of DMSO were used as controls. Subsequently, cells were stimulated with LPS (10 µg/ml), rflaA (6.9 µg/ml and 17.25 µg/ml), rflaA (6.9 µg/ml and 17.25 µg/ml) plus rOva (10 µg/ml and 25 µg/ml), and rflaA:Ova (16.9 µg/ml). Supernatants were collected 24 h post stimulation, stored at -20°C, and checked for the production of IL-6 and IL-10 by ELISA (see 3.2.6).

Experiments were repeated using C57BL/6 mDC ($3.2x10^5$ mDC/ml), additionally using the inhibitor of endocytosis chloroquine (Sigma) in the concentrations 0.01, 0.1 and 1 mM.

3.2.8 Preparation of CD4 T cells

Splenic $CD4^+$ T cells were isolated from Ova-immunized BALB/c, DO11.10 (BALB/c) and OT-II (C57BL/6) mice using the CD4 T Cell Isolation Kit from Miltenyi Biotec. CD4 T cell purity was checked by FITC-conjugated anti mouse CD4 antibody (Miltenyi Biotech, see 3.2.5) and routinely exceeded 95% (see 4.2.9). T cells ($8x10^5$ cells/ml) were co-cultured with BALB/c, C57BL/6, $MyD88^{-/-}$ (C57BL/6), $Trif^{-/-}$ (C57BL/6), and $MyD88^{-/-}Trif^{-/-}$ (C57BL/6) ($3.2x10^5$ cells/ml) in 24-well plates (Nunc), and stimulated with equimolar amounts of rOva,

rflaA, rflaA plus rOva, rflaA:Ova, rflaA:Ova (R/A), and LPS for 24 to 72 h. Supernatants were stored at -20°C and concentrations of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17A, IL-23, and IFN-γ in the culture supernatants were determined by ELISA (see 3.2.6).

3.2.9 Neutralisation assay

In order to neutralize rflaA:Ova-induced IL-10 production by BALB/c and C57BL/6 mDC $3.2x10^5$ cells/ml were seeded in 24-well plates (Nunc), stimulated with rflaA:Ova (16.9 µg/ml), and co-cultured with $8x10^5$ DO11.10 or OT-II $CD4^+$ T cells/ml, respectively in the presence of either recombinant mIL-10 (2 ng/ml) or blocking anti-mIL-10 antibody (0.1 to 10 µg/ml, both eBiosciences) for 72 h. Supernatants were stored at -20°C, and checked for the induction of IL-4, IL-10, and IFN-γ by ELISA (see 3.2.6).

3.3 Prophylactic and therapeutic intervention in the Ova-induced intestinal allergy model

3.3.1 The model of Ova-induced intestinal allergy

To investigate the immune modulating properties of the different constructs *in vivo* a model of severe Ova-induced intestinal allergy was used. In this model BALB/c mice are sensitized with Ova (50 µg Ova, Grade V, Sigma) adsorbed to aluminium hydroxide (1 mg, Pierce, absorbed for 30 minutes, RT, on a rollshaker at medium speed) by two i.p.-injections two weeks apart. Subsequently mice are continuously challenged for 5.5 to 8 days with Ova-containing food pellets (Ssniff, Soest, according to Burggraf, *in revision*). In Ova-sensitized mice Ova-pellet challenge results in disease symptoms such as ruffed fur, reduced mobility, a drop in core body temperature, soft faeces, and a distinct weight loss (Burggraf *et al., in revision*). These symptoms are most pronounced on days 4.5 to 7 of Ova-pellet challenge which characterize the most acute phase of the disease.
This model was applied to test whether prophylactic and therapeutic vaccination with either Ova, rflaA, flaA plus Ova, or the rflaA:Ova fusion protein was sufficient to prevent or treat the allergic response, respectively.

3.3.2 Prophylactic vaccination

For prophylactic vaccination, BALB/c mice were treated with equimolar amounts of rflaA (6.9 µg), Ova (10 µg, Grade V, Sigma), rflaA (6.9 µg) plus Ova (10 µg), rflaA:Ova (16.9 µg), or PBS applied twice by i.p.-injection in 1 week intervals, or with 16.9 µg rflaA:Ova applied three times i.n. under ketamin/rompun anaesthesia in 3 days intervals. All i.p.-injections were performed in a total volume of 200 µl sterile PBS, i.n.-application in a total volume of 30 µl sterile PBS (Figure 5).
The model of Ova-induced intestinal allergy was used according to Burggraf et al. (see 3.3.1). One week after the last vaccination, mice were sensitized to Ova twice in two week intervals by i.p.-injection of 50 µg Ova (Grade V, Sigma) absorbed in 1 mg aluminium-hydroxide (Pierce) in 200 µl sterile PBS (30 minutes incubation, RT, on a rollshaker at medium speed). Two weeks after the second sensitization all animals underwent continuous challenge for 5.5 to 8 days with either Ova-containing food pellets (Ssniff) or normal food for control groups (see table 1). Blood samples were collected 1 week after each sensitization (days 7 and 21) from the tail vein and after Ova-pellet challenge by cardiac puncture under deep ketamin/rompun anaesthesia.

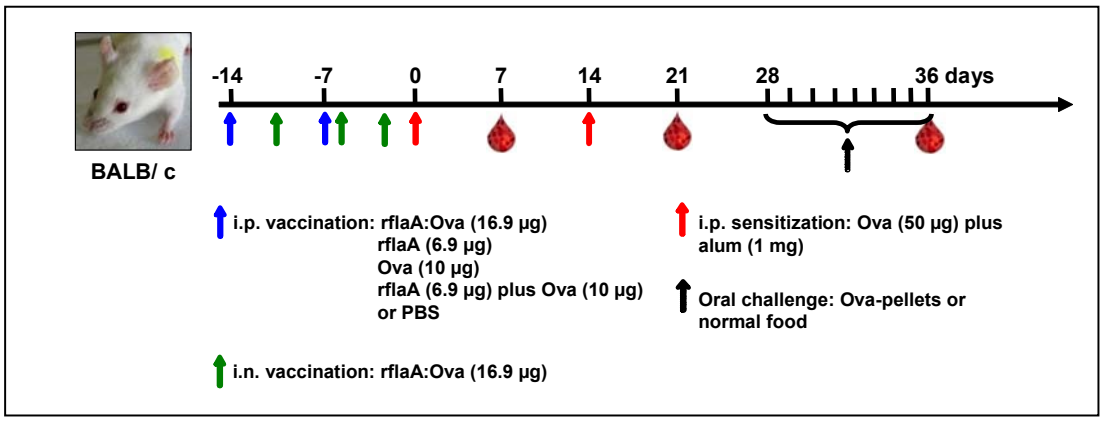


Figure 5: Vaccination scheme: prophylactic vaccination with rflaA:Ova. All constructs were applied in a final volume of 200 µl PBS. i.n.-application was performed in a total volume of 30 µl sterile PBS.

Table 1: Groups for prophylactic vaccination. NF = normal food.

Group	Prophylactic treatment	Sensitization	Challenge
PBS -> PBS -> NF	PBS i.p.	PBS	normal food
PBS -> Ova/A -> Ova	PBS i.p.	Ova plus Alum	Ova-pellets
Ova -> Ova/A -> NF	Ova i.p.	Ova plus Alum	normal food
Ova -> Ova/A -> Ova	Ova i.p.	Ova plus Alum	Ova-pellets
rflaA -> Ova/A -> Ova	rflaA i.p.	Ova plus Alum	Ova-pellets
rflaA + Ova -> Ova/A -> Ova	Ova i.p. plus rflaA i.p.	Ova plus Alum	Ova-pellets
rflaA:Ova -> Ova/A -> Ova (i.p.)	rflaA:Ova i.p.	Ova plus Alum	Ova-pellets
rflaA:Ova -> Ova/A -> Ova (i.n.)	rflaA:Ova i.n.	Ova plus Alum	Ova-pellets

3.3.3 Therapeutic vaccination

For therapeutic treatment of Ova-induced intestinal allergy BALB/c mice were first sensitized to Ova by i.p.-injection of 50 µg Ova (Grade V, Sigma) absorbed to 1 mg aluminium-hydroxide (Pierce) twice in two week intervals (Figure 6). On days 21 and 28 mice were treated by i.p.-injection of equimolar amounts of Ova (10 µg, Grade V; Sigma), rflaA (6.9 µg), rflaA (6.9 µg) plus Ova (10 µg), rflaA:Ova (16.9 µg), and PBS (200 µl). One week after the second vaccination mice were continuously challenged for 5.5 to 8 days with Ova-containing food pellets (Ssniff), whereas control groups received normal food. Blood samples were collected 1 week after each sensitization (days 7 and 21) from the tail vein and after Ova-pellet challenge by cardiac puncture under deep ketamin/rompun anaesthesia.

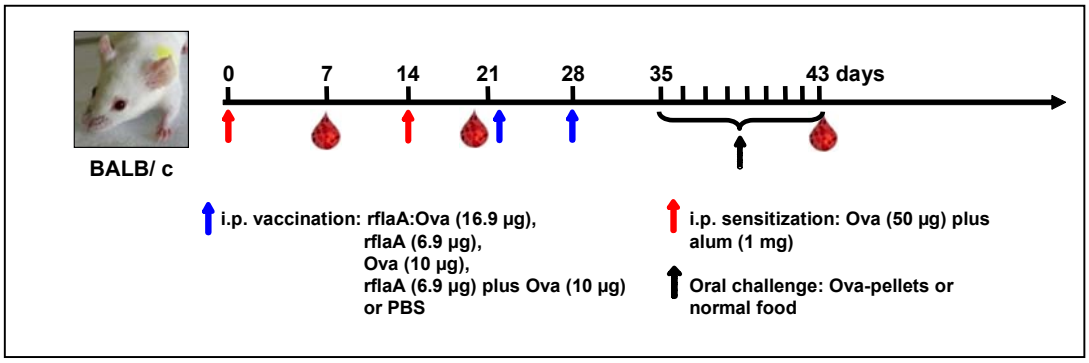


Figure 6: Vaccination protocol: therapeutic vaccination with rflaA:Ova. All constructs were applied in a total volume of 200 µl sterile PBS.

Table 2: Groups for therapeutic vaccination. NF = normal food.

Group	Sensitization	Therapeutic treatment	Challenge
PBS -> PBS -> NF	PBS	PBS i.p.	normal food
PBS -> Ova/A -> Ova	Ova plus Alum	PBS i.p.	Ova-pellets
Ova -> Ova/A -> Ova	Ova plus Alum	Ova i.p.	Ova-pellets
rflaA -> Ova/A -> Ova	Ova plus Alum	rflaA i.p.	Ova-pellets
rflaA + rOva -> Ova/A -> Ova	Ova plus Alum	Ova i.p. plus rflaA i.p.	Ova-pellets
rflaA:Ova i.p. -> Ova/A -> Ova	Ova plus Alum	rflaA:Ova i.p.	Ova-pellets

3.3.4 Proliferation assay

3.3.4.1 Preparation of antigen presenting cells

Antigen presenting cells were prepared from untreated BALB/c mice. Therefore, spleens were removed under sterile conditions and placed in 5 ml washing buffer (see table 10) in a 6 cm petri dish. Spleens were milled with the plunger of a syringe to disrupt spleens. Subsequently, 5 ml washing buffer containing the cells was filtered through a 70 µm cell strainer (BD Biosciences,) into a 50 ml falcon tube (BD Biosciences). Cells were collected by centrifugation (5 minutes, 226 g, 4°C) and transferred to a 15 ml Falcon tube (BD Biosciences), washed twice by adding 12 ml washing medium and centrifugation (RT, 5 minutes, 226 g), taken up in 5 ml washing medium, and the cell number was determined. To disrupt APC-proliferation, $1x10^8$ cells/ml were treated for 30 minutes with 50 µg/ml mytomycin C (Sigma) at 37°C. Subsequently, mitomycin C treated cells were washed three times with 10 ml washing medium (5 minutes, 226 g, 4°C), the cell pellet was resuspended in 5 ml proliferation medium (see tabe 10), cell number was determined, and adjusted to $8*10^6$ cells/ml. APC were kept on ice until needed.

3.3.4.2 Preparation of CD4 T cells from spleens and mesenterial lymph nodes

Spleens and mesenterial lymph nodes (MLN) were removed under sterile conditions and treated as described in 2.3.4.1. Red blood cells were lysed by adding 5 ml red blood cell lysis buffer (Invitrogen) and incubation for 1 to 2 minutes at room temperature. The reaction was stopped by adding 10 ml washing buffer (see table 10) and cells were washed twice by adding 12 ml washing buffer and centrifugation (226 g, 5 minutes, RT). For magnetic cell sorting cells were resuspended in 5 ml MACS-buffer (see table 10) and cell number was determined. Briefly, $CD4^+$ T cells were isolated from $4x10^7$ spleen/MLN cells using the CD4 T Cell Isolation Kit according to the manufacturers' recommendations (Miltenyi Biotec). $CD4^+$ T cell purity was checked by flow cytometry (see 3.2.5), and routinely exceeded 95%. MACS-sorted $CD4^+$ T cells were collected by centrifugation and taken up in proliferation medium (see table 10). CD4 T cell concentration was adjusted to $2x10^6$ cells/ml using proliferation medium.

3.3.4.3 CD4 T cell proliferation assay

For proliferation assays $1x10^5$ $CD4^+$ T cells and $4x10^5$ APCs were co-cultured in 96-well plates (Nunc) in a volume of 100 µl proliferation medium (containing 10% FCS) and stimulated with 100 µl of the appropriate antigen (diluted in proliferation medium without FCS) in triplicates. Concanavalin A (Sigma) was applied as positive control. After cultivating cells for 72 h (37°C, 5% CO_2, 95% humidity) 100 µl of the supernatant from each well were transferred to a fresh 96-well plate (Nunc) and stored at -20°C for cytokine measurements by ELISA (see 3.2.6). To the remaining cells 0.5 µCi of ^{3}[H]-thymidine (10 µl of a 50 µCi/ml ^{3}[H]-thymidine solution in proliferation medium without FCS, GE Healthcare) were added and cells were incubated for another 20 h (37°C, 5% CO_2, 95% humidity). Plates were stored at -20°C until measurement of ^{3}[H]-thymidine incorporation. Therefore, plates containing ^{3}[H]-thymidine labelled cells were thawed and cells were transferred to "printed filtermats" (Wallac, Finland) using a harvester (Innotech, Switzerland). Medium was discarded. Membranes were washed with water, followed by ethanol and subsequently dried at room temperature for 8 h. Membranes were covered with "melt-on wax scintillator sheets" (Wallac) and "heat sealing paper" (Wallac) and shrink-wrapped in sample bags (Wallac). Measurement of thymidine uptake was performed using a 1450 Microbeta Liquid Scintillation and Luminescence counter (Perkin Elmer, Rodgau). Data were analyzed using Excel (Microsoft).

Levels of IL-4, IL-10, and IFN-γ in the supernatants were determined using the BD Biosciences ELISA kits (BD Biosciences).

3.3.5 Staining of regulatory T cells

For staining of regulatory T cells in spleens and MLN of vaccinated and control mice the Mouse Regulatory T Cell Staining Kit was used according to the manufacturers' recommendations (eBiosciences). $1x10^6$ Splenocytes or MLN cells were stained using FITC-conjugated anti-mouse CD4, APC-conjugated anti-mouse CD25, and PE-conjugated anti-mouse Fox p 3 mAbs (antibody amounts used are indicated in table 8). 10^5 CD4 positive T cells were recorded, and the number of $CD4^+CD25^+$Fox p 3^+ T_{reg} cells was compared between the different treatment groups and untreated mice as control.

3.3.6 Determination of Ova-specific IgG1, IgG2a and IgE titers

For determination of Ova-specific IgE, IgG1 and IgG2a titers 96-well Maxisorp plates (Nunc) were coated with 50 µl per well of a 100 µg/ml Ova (Grade V, Sigma) in coating buffer (50 mM Na_2Co_3, pH 9.6) over night at 4°C. Subsequently, plates were washed three times with 200 µl/well PBST 0.05% and blocked with 200 µl/well 2% BSA in PBS for 1 h at room temperature. After another washing step (three times with 200 µl/well PBST 0.05%), 50 µl of the diluted serum samples were added. For determination of IgG1 and IgG2a levels serum samples (1:100) were used for seven serial 1:10 dilutions ($1:10^3$ – $1:10^9$). For measuring Ova-specific IgE-levels sera were diluted (1:10 and 1:50) and used for six serial 1:10 dilutions ($1:5x10^2$ – $1:5x10^7$). After incubation over night at 4°C plates were washed and incubated for 1 h with 50 µl/well of the respective Ig-isotype specific antibody (IgG1: rabbit anti mouse IgG1-HRP, IgG2a: rabbit anti mouse IgG2a-HRP, both Zytomed, Berlin, diluted 1:4000 in PBST 0.05% plus 1% BSA; IgE: biotin anti mouse IgE, BD Biosciences, diluted 1:1000 in PBST 0.05% plus 1% BSA). For determination of IgE titers plates were washed once more and incubated with 50 µl/well streptavidin-HRP (BD Biosciences, diluted 1:2000 in PBST 0.05% plus 1% BSA) for 30 minutes at room temperature. Subsequently, plates were washed six times with 200 µl/well PBST 0.05% and 100 µl/well TMB substrate solution (0.525 mM TMB, 0.01% H_2O_2 dissolved in 0.21 M potassium citrate buffer pH 3.95) were added and incubated for up to 30 minutes. The reaction was stopped by addition of 50 µl/well 25% hydrogen peroxide and absorbance (A=450 nm) was analyzed using a SpectraMAX340PC (Molecular Devices). Data were analyzed using Excel (Microsoft).

3.3.7 Quantification of Ova-specific IgG1, IgG2a and IgE levels in mouse sera

For absolute quantification of IgG1, IgG2a and IgE in mouse sera ELISA plates (Greiner Bio-One, Solingen-Wald) were coated with 5 µg/well Ova (Ova Grade V, Sigma,) in coating buffer (50 mM NaCO3, pH 9.6) over night at 4°C. For IgE-plates standards were coated with 50 µl purified rat anti mouse IgE (clone: R35-72, BD Biosciences) diluted 1:1000 in coating buffer over night at 4°C. On the next day plates were washed three times with 200 µl/well PBST 0.05% and blocked for at least 1 h using 130 µl PBS 2% BSA (Sigma). For preparation of IgE-standards monoclonal anti-dinitrophenyl antibody (clone: SPE-7, Sigma) was diluted 1:1000 in PBS 10% FCS and subsequently nine serial 1:2 dilutions were performed. For IgG1 and IgG2a standards monoclonal anti-Ova (clone: A6075, conc: 1mg/ml, Sigma) was first diluted 1:250.000 before nine serial 1:2 dilutions were performed. Serum samples were diluted 1:200 to 1:800 (IgE, IgG2a) or 1:24.000 to 1:32.000 (IgG1) in PBS 10% FCS. 50 µl of sample and standards were incubated at 4°C over night (IgE) or for 2 h at room temperature (IgG1, IgG2a). Subsequently, plates were washed three times with 200 µl/well PBST 0.05% and detected with 50 µl secondary antibody diluted in PBS 10% FCS (IgE: biotin rat anti mouse IgE, clone: R35-118, BD Biosciences, IgG1: goat anti mouse IgG1 γ1 HRP, 1 mg/ml, #A10551, IgG2a: rabbit anti mouse IgG2a HRP, #610220, both Invitrogen). After another washing step and 30 minutes incubation with 50 µl per well streptavidin-HRP (BD Biosciences, diluted 1 to 2000 in PBS 10% FCS) plates were washed six times with 200 µl/well PBST 0.05%. Subsequently, 100 µl/well TMB substrate solution were added (TMB Substrate Solution, BD Biosciences) and incubated for up to 30 minutes. The reaction was stopped by addition of 50 µl/well 25% hydrogen peroxide and absobance (A=450 nm) was analyzed using a SpectraMAX340PC (Molecular Devices). Data were analyzed using Excel (Microsoft) and GraphPad Prism 4.03 (GraphPad Software, Inc., USA).

3.3.8 Multiplex analysis of cytokine level in sera

Determination of cytokine levels in the sera of vaccinated and control mice was performed using undiluted serum samples and the FlowCytomix™ Multiplex Kit according to the manufacturer´s recommendations (Bender Medsystems, Vienna). Results were analyzed using the FlowCytomix™ Pro 2.3 software (Bender Medsystems) and processed using GraphPad Prism 4.03 (GraphPad Software, Inc., USA). Median values were computed for each treatment group.

3.3.9 Determination of cytokine levels in intestinal homogenates

On day 5.5, 6.5, or 8 of Ova-pellet challenge (see 3.3.2 and 3.3.3) mice were sacrificed and 15 cm of small intestine were removed 5 cm after the stomach. The intestines were rinsed with ice-cold PBS to remove faeces; tissues were cut in approximately 1 cm long pieces, briefly dried on filter paper, and immediately frozen in liquid nitrogen. For preparation of lysates tissues were ground to powder using pestle and mortar cooled with dry ice. Powders were dissolved in 15 ml Falcon tubes (BD, Biosciences) by adding 2 ml of ice-cold PBS plus protease inhibitor (Roche) and extensive vortexing. Lysates were centrifuged at 4000 g, 4°C for 10 minutes and the soluble fraction was transferred to 2 ml Eppendorf tubes (Eppendorf, Hamburg) and centrifuged at 10000 g, 4°C for 10 minutes. Supernatants were transferred to fresh 2 ml Eppendorf tubes (Eppendorf, Hamburg) and stored at -20°C. Overall protein concentrations in lysates were determined by BCA (Pierce, see 3.1.5) and protein concentrations were adjusted to 5 mg/ml and subjected to cytokine determination (IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-18, IL-22, IL-23, IFN-γ, MCP-1, GM-CSF, TNF-α) by multiplex ELISA (50 µl undiluted lysate per well, see 3.3.8).

3.4 Statistical analysis

Comparison between different treatment groups was performed by means of a mixed linear model with fixed factor *treatment group* and random factor *assay* (up to 3 assays with each two replicates). Confidence intervals for the estimated differences between treatment groups as well as p-values were either adjusted using the Wilcoxon signed-rank test (only for comparison of symptom scores) or the Bonferroni method (all other tests) in order to restrict the overall type I error α (false positive results i.e. false significant differences) to 5%. P-values <0.05, <0.01, and <0.001 were designated with *, **, and *** respectively.

3.5 Oligonucleotides

Table 3: Oligonucleotides used for cDNA-cloning and colony PCR. All oligonucletides were purchased from Eurofins MWG Operon (Ebersberg). Underlined: extended sequence of the respective fusion partner; *italic letters*: restriction enzyme cutting sites; **bold letters:** stop-codon; rAra h 2 protein was provided by Sonja Wolfheimer according to the protocol by Lehmann et al. (38). rPru p 3 protein was provided provided by Sven Pokoj produced in *P. pastoris* according to the protocol published in (174).

primer	primer Sequence	T_m
Colony PCR		
forward (T7 promoter)	5′-attatgctgagtgatatcccgc-3′	50°C
reverse (SP6 promoter)	5′-taagatatcacagtggattta-3′	
flaA		
forward	5′-*catatg*aaagtaaatactaatatcattagcttgaaaacacaagaatatc-3′	41-51°C
reverse	5′-*ctcgag***taa**gctgttaattaattgag-3	
Ova		
forward	5′-tatatata*catatg*atgggctccatcggcgc-3’	40°C
reverse	5′-tatatata*ctcgag***tca**ttaaggggaaacacatctgcc-3’	
flaA:Ova (flaA extend)		
forward	5′-*catatg*aaagtaaatactaatatcattagcttgaaaacacaagaatatc-3′	
reverse	5′-gctgcgccgatggagcccatgctgttaattaattgagtt-3’	40°C
flaA:Ova (Ova extend)		
forward	5′-aactcaattaattaacagcatgggctccatcggcgcagc-3′	
reverse	5′-*ctcgag***tca**ttaaggggaaacacatctgcc-3′	40°C
flaA:Ova (fusion PCR)		
forward	5′-*catatg*aaagtaaatactaatatcattagcttgaaaacacaagaatatc-3′	
reverse	5’-*ctcgag***tca**ttaaggggaaacacatctgcc-3′	55°C
flaA:Ara h 2 (flaA extend)		
forward	5′-tatata*catatg*aaagtaaatactaatatcattagcttgaaaacacaagaatatc-3′	
reverse	5′-cctgcagttcccactgctggcggctgttaattaattgagtt-3′	40°C
flaA:Ara h 2 (Ara h 2 extend)		
forward	5′-aactcaattaattaacagccgccagcagtgggaactgcagg-3′	
reverse	5′-*ctcgag***tca**atagcgatcgcggccgctatata-3′	40°C
flaA:Ara h 2 (fusion PCR)		
forward	5′-tatata*catatg*aaagtaaatactaatatcattagcttgaaaacacaagaatatc-3′	
reverse	5′-*ctcgag***tca**atagcgatcgcggccgctatata-3′	48°C
flaA:Pru p 3 (flaA extend)		
forward	5′-*catatg*aaagtaaatactaatatcattagc-3′	
reverse	5′-gcaaatgttaactcaattaattaacagcataacatgtggccaagtgtccagc-3′	41-56°C
flaA:Pru p 3 (Pru p 3 extend)		
forward	5′-gcaaatgttaactcaattaattaacagcataacatgtggccaagtgtccagc-3′	
reverse	5′-ccaccaactgcgccaccgtgaag**tga***ctcgag*-3′	41-56°C
flaA:Pru p 3 (fusion PCR)		
forward	5′-*catatg*aaagtaaatactaatatcattagc-3′	
reverse	5′-ccaccaactgcgccaccgtgaag**tga***ctcgag*-3′	63°C

3.6 Chemicals

Table 4: Chemicals, media, and proteins

description	distributor
acetic acid	Merck, Darmstadt
acetone	Merck, Darmstadt
acrylamide/bisacrylamide	Carl Roth, Karlsruhe
deoxyadenosine triphosphate (dATP)	Fermentas, St. Leon-Rot
agarose	Peqlab, Erlangen
aluminium hydroxide (Alum)	Pierce, Solingen
ammonium chloride	Sigma-Aldrich, Steinheim
ammonium persulfate	Sigma-Aldrich, Steinheim
ampicillin (Amp)	Carl Roth, Karlsruhe
antifoam B emulsion	Sigma-Aldrich, Steinheim
bafilomycin A1 (Baf A1)	Sigma-Aldrich, Steinheim
benzonase	Novagen, Darmstadt
beta-mercaptoethanol (β-ME)	Sigma-Aldrich, Steinheim
blasticidine (Bla)	InvivoGen, France
bromphenol blue	Merck, Darmstadt
bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim
carbenicillin (Carb)	Carl Roth, Karlsruhe
chloroform	Merck, Darmstadt
chloroquine	Sigma-Aldrich, Steinheim
concanavalin A (ConA)	Sigma-Aldrich, Steinheim
cytochalasin D (Cyt D)	Sigma-Aldrich, Steinheim
Dulbecco/Vogt modified Eagle's minimal essential medium (DMEM)	Invitrogen, Karlsruhe
dimethyl sulfoxide (DMSO)	Fluka, Neu-Ulm
dithiothreitol (DTT)	Molecula, UK
ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Steinheim
ethanol	Merck, Darmstadt
ethidium-bromide (EtBr)	Fluka, Neu-Ulm
fetal calf serum (FCS)	Invitrogen, Karlsruhe Biochrome, Berlin
Fms-related tyrosine kinase 3 ligand (Flt-3L)	R&D Systems, Wiesbaden
formaldehyde	Merck, Darmstadt
glycerine	Sigma-Aldrich, Steinheim
glycine	Sigma-Aldrich, Steinheim
granulocyte macrophage colony-stimulating factor (GM-CSF)	R&D Systems, Wiesbaden
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Merck, Darmstadt
hydrogen peroxide	Sigma-Aldrich, Steinheim
imidazol	Merck, Darmstadt

iodacetamide (IAA)	Merck, Darmstadt
description	**distributor**
sopropyl β-D-1-thiogalactopyranoside (IPTG)	Carl Roth, Karlsruhe
isoflurane	Baxter, Unterschleißheim
ketamin/rompun	Bayer, Leverkusen
lysogeny broth-medium (LB-medium)	Carl Roth, Karlsruhe
L-glutamine	Invitrogen, Karlsruhe
lysozyme	Boehringer, Mannheim
magnesium chloride	Merck, Darmstadt Fermentas, St. Leon-Rot
modified Eagle's minimal essential medium (MEM)	Invitrogen, Karlsruhe
methanol	Merck, Darmstadt
mitomycin C	Sigma-Aldrich, Steinheim
nickel nitrilotriacetic acid-agarose (Ni-NTA-agarose)	Quiagen, Hilden
ovalbumin (grade V) (Ova)	Sigma-Aldrich, Steinheim
ovalbumin (grade VII) (Ova)	Sigma-Aldrich, Steinheim
paraformaldeyd	Sigma-Aldrich, Steinheim
penicillin/streptomycin (Pen/Strep)	Invitrogen, Karlsruhe
phenol:chloroform:isoamylalcohol (25:24:1)	Sigma-Aldrich, Steinheim
protease inhibitor	Roche, Grenzach-Wyhlen
proteinase K	Sigma-Aldrich
recombinant mouse IL-10 (rmIL-10)	eBiosciences, USA
Roswell Park Memorial Institute medium 1640 (RPMI 1640)	Invitrogen, Karlsruhe
sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Steinheim
sarcosyl-lauryl-sulfate (SLS)	Novagen, Darmstadt
super Optimal Broth-medium (SOC-medium)	Invitrogen, Karlsruhe
sodium acetate	Merck, Darmstadt
sodium azide	Fluka, Neu-Ulm
sodium chloride	Merck, Neu-Ulm
sodium pyruvate	Sigma-Aldrich, Steinheim
tetramethylethylenediamine (TEMED)	Sigma-Aldrich, Steinheim
[methyl-^{3}H] thymidine	GE Healthcare, Munich
tetra-methyl benzidine (TMB)	Carl Roth, Karlsruhe
tris/HCl	Merck, Darmstadt
triton X-100	Sigma-Aldrich, Steinheim
trypan blue	Carl Roth, Karlsruhe
trypsin-EDTA	Cambrex, Belgien
Tween®20	Sigma-Aldrich, Steinheim
urea	Merck, Darmstadt

3.7 Consumables and Equipment

Table 5: Consumables

description	distributor
cell culture flasks	Nunc, Wiesbaden
cell culture plates	Nunc, Wiesbaden
cell strainer, 70 µm	BD Biosciences, Heidelberg
canulaes	Braun, Melsungen
cryo tubes	Nunc, Wiesbaden
chelate spin columns	BD Biosciences, Heidelberg
ELISA plates: Maxisorp Microlon, high binding	 Nunc, Wiesbaden Greiner Bio-One, Solingen-Wald
Eppendorf tubes	Eppendorf, Hamburg
FACS tubes	BD Biosciences, Heidelberg
Falcon tubes	BD Biosciences, Heidelberg
glassware	Schott, Mainz
heat sealing paper	Wallac, Finland
LS columns	Miltenyi Biotech, Bergisch-Gladbach
MeltiTex™ A Melt-on Scintillator Sheets	Wallac, Finland
nitrile gloves	Braun, Melsungen
plastic cuvettes	Roth, Karslruhe
pipette tips	Eppendorf, Hamburg Thermo Scientific, Dreieich Greiner BioOne, Solingen
printed filtermat A	Wallac, Finland
sample bag	Wallac, Finland
single use syringes	Braun, Melsungen
spandex gloves	Braun, Melsungen
sterile filters	Merck, Darmstadt
silica glass cuvettes	Hellma, Mühlheim
syringe filters	Minisart, Baar

Table 6: Equipment

description	distributor
analytical balance (Atilon)	Acculab, Göttingen
autoclave (H+P varioclave)	H&P, Oberschleißheim
CO_2-incubator (Hera cell 150)	Heraeus Sorvall, Hanau
ELISA-Reader (Spectra MAX340PC)	Molecular Devices, USA
Electrophoresis: DNA proteins (Mini Protein III) power supply (Power Pack P25)	 RAGE, USA BioRad, Munich Biometra, Goettingen
benchtop fermenter, 2 litre (Boro 2.2)	Schott, Mainz
flow cytometer (BD LSRII)	BD Biosciences, Heidelberg
FPLC hardware (Äkta FPLC)	Amersham, Freiburg
FPLC columns: XK-16 (IMAC) HisTrap FF crude (IMAC) Hiload™ XK16/60 Superdex 200 prep grade (SEC)	 Amersham, Freiburg GE healthcare, Munich Amersham, Freiburg
fridge-freezer	New Brunswick, Neu-Isenburg Heraeus Sorvall, Hanau Liebherr, Biberach Bosch, Gerlingen
harvester for ^{3}H-thymidine incorporation assay	Innotech, Switzerland
laminar airflow cabinet: BSB6a LaminAir H2448K SterilGARD typeA/B3	 GelAire, Australia Heraeus Sorvall, Hanau Baker, USA
medical instruments	Sigma-Aldrich, Steinheim
microscopes	Zeiss, Oberkochen
microwaves	Privileg, Fürth
mortar	Haldenwanger, Waldkraiburg
heating shaker (Thermomixer 5436, 5437)	Eppendorf, Hamburg
pestle	Haldenwanger, Waldkraiburg
pipettes: Pipettboy, Acu Research Pro Finnpipette	 IBS, Switzerland Eppendorf, Hamburg Thermo Scientific, Dreieich
pH-meter (pH level 1)	InoLab, Weilheim
photometer: UV-1202 GENEQUANT Pro	 Shimadzu, Duisburg Amersham, Freiburg
endotoxin detection (Endosafe PTS)	Charles River, Sulzfeld
scintillation counter (1450 Microbeta Liquid Scintillation and Luminescence counter)	Perkin Elmer, Rodgau
stirring heating plate (MR 3001)	Heidolph, Kelheim
spektropolarimeter (J-810 S)	Jasco, Groß-Umstadt
shaking incubator (Innova 4335/44)	New Brunswick, Neu-Isenburg

description	distributor
thermocycler: GeneAmp PCR system 2700 Mastercycler Gradient	 Biometra, Göttingen Eppendorf, Hamburg
ultrasonic device (Sonoplus)	Bandelin, Berlin
UV-decomentation station	Intas, Göttingen
vortexer (MS1 minishaker)	IKA, Staufen
washer for mitrotiterplates (ELX405)	Bio-Tek, Neufahrn
water bath	Köttermann, Uetze/Hänigsen GFL, Burgwedel
centrifuges: 5415C 5415R Multifuge 3SR Megafuge 1.0R EvolutionRC RC 5B Plus	 Eppendorf, Hamburg Eppendorf, Hamburg Heraeus Sorvall, Hanau Heraeus Sorvall, Hanau Heraeus Sorvall, Hanau Heraeus Sorvall, Hanau

Table 7: Commercially available systems

description	distributor
BCA Protein Assay Kit (Pierce)	Thermo Scientific, Solingen
CD4 TC Isolation Kit	Miltenyi, Bergisch-Gladbach
DNA-marker: 100 bp ladder (SM1343) 1 kb plus ladder Generuler 1 kb plus ladder	 New England Biolabs, Frankfurt Invitrogen, Darmstadt Fermentas, St. Leon-Rot
dNTP mix 10 mM	Fermentas, St. Leon-Rot
ELISA kits BD OptEIA™ ELISA Sets Ready-SET-GO! ELISA kits FlowCytomix™ multiplex ELISA	 BD Biosciences, Heidelberg *e*Bioscience, USA BenderMedsystems, Vienna
fixation/permeabilization kit	BD Biosciences, Heidelberg
GelCode Blue	Pierce, Solingen
gel extraction kit	Quiagen, Hilden
IMAC-zentrifugation columns (Vivapure Metal Chelate Mini/Maxi)	Vivascience, Sartorius group, Hannover
Mouse Erythrocyte Lysing Kit	R&D Systems, Wiesbaden
Mouse Regulatory T Cell Staining Kit	Miltenyi Biotech, Bergisch-Gladbach
PCR-Master Mix	Roche, Mannheim
PCR Purification Kit	Roche, Mannheim
PCR®4 TOPO cloning kit	Invitrogen, Darmstadt
pET15b	Novagen, Darmstadt
pET32a	Novagen, Darmstadt
pGEM® T easy	Promega, Mannheim
quick ligation kit	New England Biolabs, Frankfurt
plasmid Maxi / Mini Kit	Quiagen, Hilden
Platinum Taq Polymerase	Invitrogen, Darmstadt
Ponceau S staining	Fluka, Neu-Ulm
Protein Refolding Kit	Novagen, Darmstadt
protein Marker: SeaBlue Plus 2, prestained	 Invitrogen, Darmstadt
red blood cell lysis buffer	Sigma-Aldrich, Steinheim Invitrogen, Darmstadt
restriction enzyme sets	New England Biolabs, Frankfurt
spin-X zentrifugation system	Vitaris, Baar
T4-DNA-ligase set	New England Biolabs, Frankfurt
TMB substrat reagent set	BD Biosciences, Heidelberg

3.8 Antibodies

Table 8: Antibodies used for flow cytometry and ELISA. A.H. = armeniam hamster.

antibody	isotype	label	µg/staining or µg/ml	distributor
rat anti mouse B220	IgG2a, κ	PeCy5	0.1	BD Biosciences
rat anti mouse CD3	IgG2b κ	FITC	0.1	Southern Biotech
rat anti mouse CD4	IgG2a κ	APC	0.2	*e*Bioscience
rat anti mouse CD4	IgG2a κ	FITC	0.25	*e*Bioscience
rat anti mouse CD4	IgG2b	FITC	5 µl (conc. unknown)	Miltenyi Biotech
hamster anti mouse CD11c	IgG1 λ2	APC	0.02	BD Biosciences
rat anti mouse CD11b	IgG2b	PB	0.1	Invitrogen
rat anti mouse CD16/32	IgG2b κ	none	1	BD Biosciences
rat anti mouse CD19	IgG2a κ	FITC	0.1	Southern Biotech
rat anti mouse CD25	IgG1, λ	APC	0.12	*e*Bioscience
rat anti mouse CD40	IgG2a, κ	PE	0.5	*e*Bioscience
A.H. anti mouse CD40	IgM, κ	FITC	0.25	*e*Bioscience
A.H. anti mouse CD69	IgG	PE	0.06	*e*Bioscience
A.H. anti mouse CD80	IgG	FITC	0.25	*e*Bioscience
A.H. anti mouse CD80	IgG	PE	0.25	*e*Bioscience
A.H. anti mouse CD86	IgG	FITC	0.25	*e*Bioscience
rat anti mouse Fox p 3	IgG2a κ	PE	0.5	*e*Bioscience
rat anti mouse IgE	IgE κ IgE	Biotin purified	0.5 µg/ml 0.5 µg/ml	BD Biosciences BD Biosciences
anti mouse dinitrophenyl (IgE standard)	IgE	purified	1 µg/ml	Sigma
rabbit anti mouse Ova goat anti mouse Ova	IgG1 IgG1	HRP HRP	0.25 µg/ml 0.5 µg/ml	Zytomed Invitrogen
anti chicken Ova	IgG1	HRP	4 ng/ml	Sigma
rabbit anti mouse Ova rabbit anti mouse Ova	IgG2a IgG2a	HRP HRP	0.25 µg/ml 1 µg/ml	Zytomed Invitrogen
rat anti mouse IL-2 rat anti-mouse IL-2	IgG2a κ IgG2b κ	none Biotin	1 µg/ml 1 µg/ml	Biolegend Biolegend
rat anti mouse IL-6	IgG1 κ	PE	0.4	*e*Bioscience
rat anti mouse IL-10	IgG2b κ	PE	0.4	*e*Bioscience
rat anti mouse IL-10	IgG2b κ	none	0.1 - 10	*e*Bioscience
isotype controls	rat IgG1 mouse IgG2a rat IgG2a rat IgG2b A.H. IgG rat IgG1 rat IgG2a A.H. IgG	PE PE PE PE PE FITC FITC FITC	0.25 0.25 0.1 0.1 0.1 0.1 0.25 0.25	*e*Bioscience *e*Bioscience *e*Bioscience *e*Bioscience *e*Bioscience BD Biosciences *e*Bioscience *e*Bioscience
mouse anti mouse MHC I	IgG2a κ	PE	0.1	*e*Bioscience
rat anti mouse MHC II	IgG2b	FITC	0.25	*e*Bioscience
mouse anti mouse TLR5	IgG2a	PE	0.5	Abcam
streptavidin-HRP		HRP	1:2T to 1:4T	BD Biosciences

3.9 Buffers

Table 9: Buffer and solutions

description	composition
CAPS-buffer	20 mM CAPS pH 11.0
DNA-sample buffer (5x)	5 ml glycerin 4.5 ml $H_2O_{zyclodest.}$ 0.5 ml TAE (1x) 1.4 mM bromo-chloro-phenol blue 1.9 mM xylencyanol
FACS-buffer	PBS 0.3% sodium azide 1% BSA 24 mM EDTA pH 8.0 sterile filtered 0.45 µm
ELISA-coating buffer	50 mM $Na_2CO_3/NaHCO_3$ pH 9.6
ELISA-incubation/dilution buffer	PBS 10% FCS
ELISA-washing buffer	PBS 0.05% Tween® 20
ELISA-HRP substrate solution	20 ml potassium citrate buffer 1 ml TMB-solution 6.6 µl 30% H_2O_2
potassium citrate buffer	229 mM citric acid 303 mM KOH ad 500 ml $H_2O_{zyclodest.}$ pH 3.95
MACS-buffer	PBS 0.5% BSA 2 mM EDTA pH 7.2
MOPS-buffer	40 mM MOPS 10 mM NaAc 1 mM EDTA 0.5 M NaCl pH 7.4
paraformaldehyde	4% paraformaldehyde in PBS
PBS	1.5 mM KH_2PO_4 0.8 mM Na_2HPO_4 137 mM NaCl 3 mM KCl pH 7.1
PCR-buffer	100 mM Tris-HCl 500 mM KCl pH 8.3
proteinase K solution	1 mg proteinase K/ml

description	composition
SDS running buffer	19.2 M glycine 5 M Tris (pH 6.8) 10% SDS
SDS staking gel buffer (4x)	0.5 M Tris pH 6.8 (adjusted with HCl)
SDS separating gel buffer (4x)	1. M Tris pH 8.0 (adjusted with HCl)
TMB-solution	20.9 mM TMB 2.5 ml acetone ad 25 ml methanol
tris-HCl	200 mM Tris HCl pH 8.5 / pH 7.5

3.10 Culture media, cell lines, and animals

Table 10: Culture media

description	composition	comment
bacterial growth medium	LB-medium 50 µg/ml ampicillin	for agar plates: 15 g/l agar-agar 100 µg/ml ampicillin
HEK cell medium	DMEM 500 ml 10% FCS 2 mM L-glutamine 180 mM penicillin 172 mM streptomycin +/- 10 µg/ml blasticidine	for assays: 2% FCS no blasticidine
DC-medium	RPMI 1640 500 ml 10% FCS for DC 2 mM L-glutamine 180 mM penicillin 172 mM streptomycin 100 mM HEPES 1 mM sodium pyruvate 0.1 mM β-mercapto-ethanol	FCS lot has to be tested carefully!
proliferation medium	RPMI 1640 500 ml 2 mM L-glutamine 180 mM penicillin 172 mM streptomycin 0.1 mM β-mercapto-ethanol	FCS is added seperately FCS lot has to be tested carefully!
washing medium	RPMI 1640 500 ml 5% FCS for BMMC 2 mM L-glutamine 180 mM penicillin 172 mM streptomycin 0.1 mM β-mercapto-ethanol	FCS lot has to be tested carefully!

Table 11: *E. coli* cloning and expression cells

description	genotype	distributor
BL21 Star DE3 chemocompetent cells	F– ompT hsdSB(rB–, mB–) gal dcm rne131 (DE3)	Invitrogen, Darmstadt
TOP10 chemocompetent cells	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	Invitrogen, Darmstadt
Origami (DE3) chemocompetent cells	Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F′[lac^+ $lacI^q$ pro] (DE3) gor522::Tn10 trxB pLysS (Cam^R, Str^R, Tet^R)	Novagen, Darmstadt

Table 12: Cell lines

cell line	description	culture medium	source of supply
HEK293	human embryonic kidney cells	DMEM	ATCC, USA
HEK293 mTLR5	HEK293 stably trans-fected with murine TLR5	DMEM + blasticidine	Invivogen, France
HEK293 hTLR5	HEK293 stably trans-fected with human TLR5	DMEM + blasticidine	Invivogen, France

Table 13: Animals used for *in vitro* and *in vivo* assays. Upon delivery, all purchased mice had an age of 6 to 8 weeks.

strain name	MHC haplotype	sex	source of supply
BALB/c	$H\text{-}2^d$	♀	Jackson, USA
C57BL/6J	$H2^b$	♀	Jackson, USA
DO11.10	$H\text{-}2^d$	♀ or ♂	PEI animal breeding facility
OT-II	$H2^b$	♀ or ♂	PEI animal breeding facility
$MyD88^{-/-}$	$H2^b$	♀ or ♂	U. Kalinke and PEI animal breeding facility
$Trif^{-/-}$	$H2^b$	♀ or ♂	U. Kalinke and PEI animal breeding facility
$MyD88^{-/-}Trif^{-/-}$	$H2^b$	♀ or ♂	U. Kalinke and PEI animal breeding facility

4. Results

4.1 Generation and quality assessment of recombinant proteins

4.1.1 cDNA-cloning and protein expression of rOva and rflaA:Ova

The Ova cDNA-sequence was amplified via PCR, and the resulting PCR product displaying the expected size of 1200 base pairs (Figure 7-1) was digested with NdeI and XhoI and ligated in to the likewise digested and purified pET15b vector. Sequence identity was verified by bidirectional Sanger sequencing. Two clones with the correct sequence were subjected to small-scale test expressions, the clone with the highest expression rate was chosen for large scale expression and purification (data not shown).

For cloning of the rflaA:Ova fusion protein, both flaA and Ova were elongated in preliminary PCR-reactions with sequence stretches complementary to the respective fusion partner (Figure 7-3 and -4) and fused in an assembly PCR using the 1:10 diluted and unpurified PCR-products of both preliminary reactions as templates (Figure 7-5). The correctly sized (approx. 2000 bp) product was purified via gel extraction to remove template molecules (Figure 7-6). The purified fusion product was A-tailed, ligated into pGEM® T easy, and transformed into *E. coli* TOP10 cells. Positive clones were selected by colony-PCR, plasmids were prepared, and digested with NdeI and XhoI. Purified inserts were ligated into the likewise digested and purified pET15b vector, and transformed into *E. coli* BL21 Star DE3 expression cells. Positive clones with an expected size of approximately 2000 bp were checked by analytical restriction digest (using NdeI and XhoI, Figure 7-7) and bidirectional Sanger sequencing. Clones with the correct cDNA sequence were subjected to small-scale test expressions, and the clone with the highest expression rate was chosen for large scale expression and purification (data not shown).

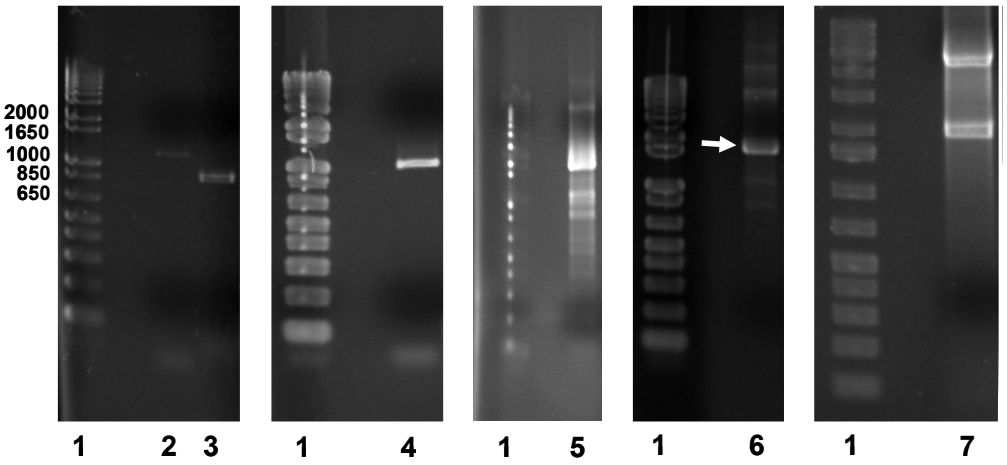


Figure 7: Cloning of Ova and flaA:Ova cDNA. (1) Marker (1 kb plus, Invitrogen), (2) Ova for pET15b, (3) flaA:Ova (rfla extended), (4) flaA:Ova (Ova extended), (5) fusion flaA:Ova, (6) gel extraction fusion flaA:Ova, (7) flaA:Ova test restriction, expected sizes: flaA:Ova (flaA extended): 884 bp, flaA:Ova (Ova extended): 1179 bp, flaA:Ova: 2034 bp, Ova: 1192 bp.

4.1.2 cDNA-cloning and protein expression of rflaA:Ara h 2

By PCR both flaA and Ara h 2 were elongated with sequence stretches complementary to the respective fusion partner (Figure 8-2 and -3). Here, both obtained cDNA sequences had the expected sizes of approximately 900 bp for the extended flaA and 450 bp for the extended Ara h 2. In an assembly PCR both sequences were fused using the 1:20 diluted and unpurified PCR-products of both preliminary reactions as templates (Figure 8-4). The resulting fusion product which displayed the expected size of approximately 1400 bp was digested with NdeI and XhoI and purified via gel extraction to remove template molecules (Figure 8-5). The purified fusion product was ligated into the likewise digested and purified pET15b vector and transformed into *E. coli* BL21 Star DE3 expression cells. Positive clones were checked by analytical restriction digest (using NdeI and XhoI) and bidirectional Sanger sequencing. Clones with confirmed cDNA fusion sequence were subjected to small-scale test expressions, and the clone with the highest expression rate was chosen for large scale expression and purification (data not shown).

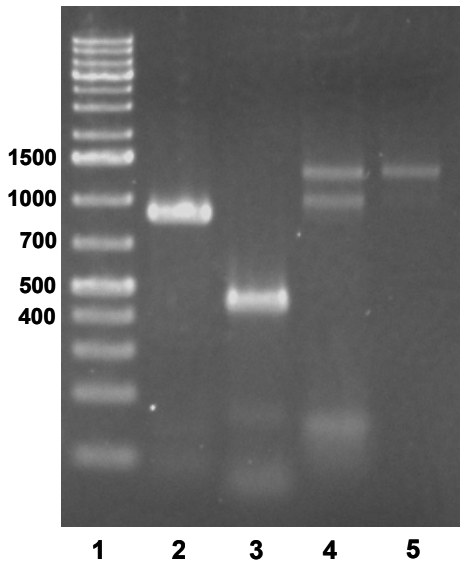


Figure 8: Cloning of rflaA:Ara h 2 into pET15b. (1) Marker (Generuler 1 kb plus), (2) flaA:Ara h 2 (flaA extended) (3) flaA:Ara h 2 (Ara h 2 extended), (4) fusion flaA:Ara h 2, (5) gel ectraction flaA:Ara h 2, expected sizes: flaA:Ara h 2 (flaA extended): 892 bp, flaA:Ara h 2 (Ara h 2 extended): 456 bp, flaA:Ara h 2: 1368 bp.

4.1.3 Generation of rflaA, rflaA:Pru p 3, rPru p 3 and rAra h 2

Recombinant *Listeria monocytogenes* flaA and flaA-Pru p 3 fusion protein (rflaA:Pru p 3) were cloned and prepared by Stefan Mende (177), recombinant Pru p 3 was provided by Sven Pokoj produced in *P. pastoris* according to the method described in (174), and rAra h 2 was prepared by Sonja Wolfheimer and Stefan Mende according to the protocol by Lehmann et al. (38).

4.1.4 Recombinant proteins can be produced with high yield and purity using E. coli

All proteins were expressed with an N-terminal His_6-tag as described in 3.1.2 and 3.1.4. The amounts of final purified proteins derived from 1 litre *E. coli* culture were 20 mg rflaA, 2.5 mg rOva, 3 mg rflaA:Ova, 6 mg rPru p 3, 5 mg rflaA:Pru p 3, 2.5 mg rAra h 2, and 5.5 mg rflaA:Ara h 2. Endotoxin was depleted by SEC to 11 pg/µg for rflaA, 0.15 pg/µg for rOva, 2.9 pg/µg for rflaA:Ova, 0.285 pg/µg for rflaA:Pru p 3, and non detectable levels for rflaA:Ara h 2 (see table 14). Recombinant Pru p 3 was expressed in and purified from the non-bacterial *P. pastoris*-system and thus did not contain endotoxins.

Table 14: Apparent molecular weight, yield, and endotoxin content of purified proteins. Endotoxin content was determined by *Limulus* amebocyte lysate (LAL) test (detection limit 0.05 EU/25 µl test solution).

construct	MW (kDa)	yield [mg/l]	expression cells	endotoxin content [pg LPS/ µg protein]
rflaA	30.5	20	*E. coli* BL21 Star DE3	11
rOva	44	2.5	*E. coli* BL21 Star DE3	0.15
rPru p 3	9.1	6	*P. pastoris*	<0.05
rAra h 2	17.5	2.5	*E. coli* Origami	0.27
rflaA:Ova	73.2	3	*E. coli* BL21 Star DE3	2.9
rflaA:Pru p 3	41.5	5	*E. coli* BL21 Star DE3	0.285
rflaA:Ara h 2	51	5.5	*E. coli* BL21 Star DE3	<0.05

Final protein preparations were checked for puritiy by gel electrophoresis. According to SDS-PAGE and Coomassie staining purity was calculated to be at least 95% for all prepared proteins (Figure 9). Whereas the flagellin containing fusion constructs rflaA:Ova, rflaA:Pru p 3, and rflaA:Ara h 2 showed partial high molecular weight aggregation products, covalent aggregates were not observed for either rflaA, rOva, rPru p 3 or rAra h 2, respectively.

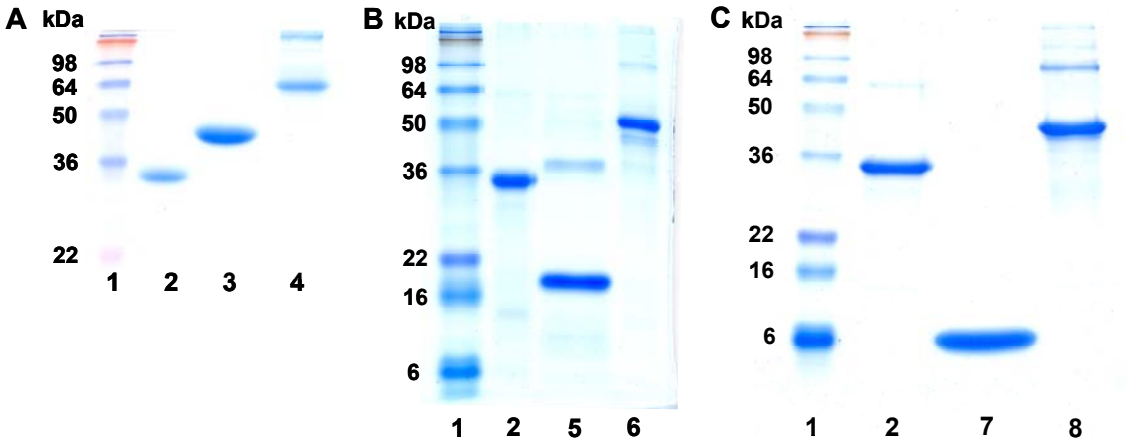


Figure 9: Recombinant proteins are produced with high purity. All constructs were cloned into pET15b and expressed using BL21 DE3 cells. After expression target proteins were purified using a two step chromatography strategy consisting of IMAC and SEC and checked for puritiy on a 15% SDS-PAGE; purification of rflaA, rOva, and rflaA:Ova (A), rflaA, rAra h 2, and rflaA:Ara h 2 (B), rflaA, nPru p 3, and rflaA:Pru p 3 (C). (1) molecular weight marker SeaBlue plus, (2) rflaA, (3) rOva, (4) rflaA:Ova (5 µg/slot), (5) rAra h 2 (4 µg/slot), (6) rflaA:Ara h 2 (4 µg/slot), (7) nPru p 3 (6 µg/slot), (8) rflaA:Pru p 3 (3 µg/slot); gel electrophoresis was performed under reducing conditions.

4.1.5 Flagellin fusion proteins aggregate due to intermolecular disulfide bonds

Under non-reducing conditions all flagellin containing fusion proteins displayed high molecular aggregation products, hampering separation by SDS gel electrophoresis (Figure 10). To analyze whether the observed aggregates in flagellin containing fusion proteins could be separated from the monomeric protein, partially purified rflaA:Ova was subjected to different types of chromatographies. Hereby, neither a combination of affinity chromatography (IMAC) and anion exchange (MonoQ) chromatography (Figure 10-2), nor size exclusion chromatography (Figure 10-3) proved capable of separating aggregates from the monomer.

Since aggregate formation was strongly reduced under reducing conditions, it is probably caused by unwanted intermolecular disulfide bonds between allergen molecules. In line with this assumption, for rflaA, which does not contain any cysteine residues, no aggregates formed by covalent binding were observed. However, analysis of aggregate formation in rflaA preparations by dynamic light scattering revealed non-covalent aggregates that were too large to be separated by chromatographic means (Himly, personal communication). To check whether the observed aggregation was caused by intermolecular disulfide bonds, rflaA:Ova was subjected to either detergents or a stable reduction of cysteine bonds by reduction and alkylation (R/A). Addition of sarcosyl-lauryl-sulfate (SLS), a mildly denaturating agent was sufficient to reduce high molecular aggregates (Figure 10-4). Of note, upon SLS-treatment of non-purified rflaA:Ova a heterogenous mixture of proteins of lower molecular weight than rflaA:Ova (<74 kDa) was detected (Figure 10-4). These might be degradation products of rflaA:Ova which were linked to the untreated aggregates via disulfide bonds and thus became visible upon SLS-mediated reduction. More likely, this appearance of undefined proteins was an artifact caused by a higher overall protein concentration within the sample used for SLS-treatment (Figure 10-4 showing a higher overall protein amount than Figure 10-2 or 3 although in all lanes 20 µl of the same protein expression were applied). In this case reliable protein quantification is not possible since these crude mixtures contain substances interfering with colorimetric protein detection.

Moreover, stable reduction of cysteine bonds by reduction and alkylation completely abrogated covalent aggregation (Figure 10-5). Therefore, the observed aggregation in flagellin:allergen fusion proteins is likely caused by intermolecular disulphide bonds between rOva, rPru p 3, or rAra h 2 molecules respectively. As a consequence, in order to minimize the degree of aggregation, for all *in vitro* and *in vivo* assays 0.03% SLS (which was shown to have no detrimental effects on flaA-mediated activation of HEK293 cells *in vitro*, data not shown) were added to rflaA:Ova. Furthermore, to examine whether the oligomerization

influenced the immune modulatory effect of the fusion construct, reduced and alkylated rflaA:Ova (R/A) was applied in all *in vitro* assays.

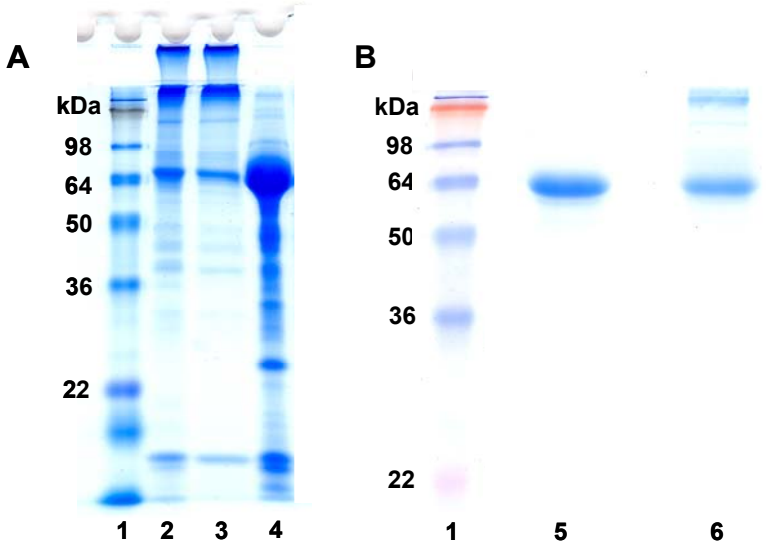


Figure 10: Recombinant rflaA:Ova fusion protein shows high molecular aggregates that can not be removed by chromatography (A) but resolved by detergents and reduction and alkylation (B). (1) marker SeaBlue plus, (2) rflaA:Ova partially purified by IMAC and MonoQ (20 µl protein solution, concentration not determined), (3) rflaA:Ova partially purified by SEC (20 µl protein solution, concentration not determined), (4) rflaA:Ova treated with SLS (20 µl non-purified protein, concentration not determined); (5) reduced and alkylated rflaA:Ova (R/A) (5 µg/slot), (6) untreated rflaA:Ova finally purified by IMAC and SEC (according to 3.1.2, 5 µg/slot), 15% SDS-PAGE performed under non-reducing conditions.

4.1.6 Recombinantly expressed proteins show a considerable amount of secondary structure

Correct folding of secondary structure elements was confirmed by CD-spectroscopy. Hereby, all proteins displayed minima at 208 and 220 nm which are typical for properly folded α-helical structures, indicating the preparation of proteins with a considerable amount of secondary structure. CD-spectra of rflaA:Ova and rflaA:Ova (R/A) were nearly identical and showed high similarity to the CD-spectrum of rflaA and rOva (Figure 11 A). These results demonstrate that reduction and alkylation has no impact on the conformation of the mainly α-helical protein rflaA (comprising no cysteine residues), and suggested that the folding of the flagellin part dominates the shape of the fusion proteins CD-spectra.

CD-spectra of rflaA:Pru p 3 and rflaA:Ara h 2 (Figure 11 B and C) displayed strong similarity to correctly folded allergen preparations, and therefore suggested considerable amounts of correctly formed secondary structure elements. The anomaly in curve progression observed between 190 and 200 nm for rflaA:Pru p 3 (Figure 11 B) is an artefact caused by the high concentrations of salts contained in the fusion protein preparation, which were required to

maintain protein solubility. In summary all recombinant proteins generated in this thesis demonstrated considerable amounts of secondary structure elements, indicating the production of properly folded molecules.

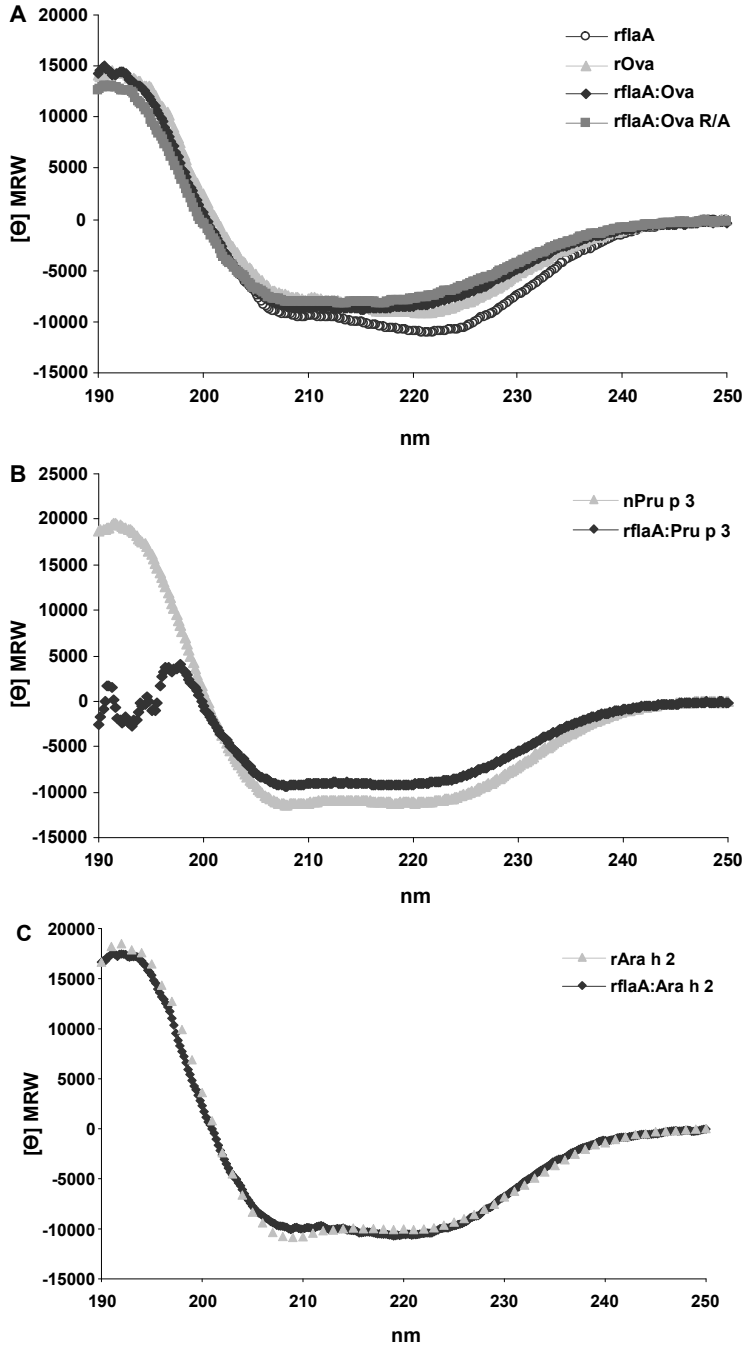


Figure 11: Recombinant allergens, flagellin A and flagellin fusion proteins display a considerable amount of secondary structure. Circular dichroism (CD)-spectra of rflaA, rOva and rflaA:Ova, rflaA:Ova (R/A) (A), CD-spectrum of rflaA:Pru p 3 compared to nPru p 3 (B), CD-spectrum of rflaA:Ara h 2 compared to rAra h 2 (C); CD-spectra were recorded using purified proteins (0.022 to 0.027 nmol/ml, 200 µg/ml), dialyzed against 10 mM phosphate buffer. Ten scans were accumulated; spectra obtained with buffer were subtracted. Results were expressed as mean residue molar ellipticity $[\Theta]_{MRW}$ and served as an indication for formation of secondary structure elements.

4.1.7 rflaA and rflaA:Ova are able to bind and activate TLR5

In order to determine the biological activity of *L.m.* rflaA and recombinant fusion proteins of flaA with Ova, Pru p 3 and Ara h 2 in respect to TLR5 activation, HEK293 wildtype (wt) and transgenic cells expressing either murine (m) or human (h)TLR5 (InvivoGen) were stimulated with the different constructs or commercially available *Salmonella typhimurium* flagellin C (rfliC, InvivoGen) used as positive control (Figure 10). Recombinant flaA and rfliC dose-dependently induced comparable levels of IL-8 secretion from TLR5 transfected HEK cells (Figure 12), but not from HEK293 wt cells, indicating recombinant *L.m.* rflaA to have a TLR5 activation capacity similar to rfliC.

When testing the different fusion proteins a special interest and emphasis was put on the rflaA:Ova construct as model protein. This was done since Ova is a widely used model antigen for immunological studies for which many tools and protocols are available. The rflaA:Ova fusion protein was able to activate the human and the murine TLR5 (Figure 12). Similar results were obtained for flagellin fusion proteins containing either Ara h 2 or Pru p 3 (Figure 13). Interestingly, although identical molar concentrations were considered when applying rflaA:allergen fusion proteins and rflaA as ligands, a reduced TLR5-dependent IL-8 secretion was observed upon stimulation with the fusion proteins. For example, the effective dose to induce a half-maximal release (ED_{50}) of IL-8 upon activation of mTLR5 was 9-times lower for flaA than for rflaA:Ova. ED_{50} for the rflaA-induced activation of hTLR5 was 4-times lower than for rflaA:Ova. Additionally for both receptors IL-8 secretion induced upon stimulation with partially defolded rflaA:Ova R/A was approximately by factor 3 lower compared to the untreated rflaA:Ova (Figure 10). Besides this reduced TLR5 activation at lower concentrations, maximal secretion of IL-8 was comparable for rflaA, rflaA:Ova, rflaA:Pru p 3 and rflaA:Ara h 2 upon stimulation of both TLR5-transgenic cell lines with protein concentrations higher than 100 µg/ml. As expected recombinant allergens Ova, rPru p 3 or rAra h 2 alone did not induce detectable IL-8 secretion, neither did stimulation of the untransfected HEK293 cells with any of the used constructs result in a detectable IL-8 production (Figure 12 and Figure 13), proving the test systems specificity.

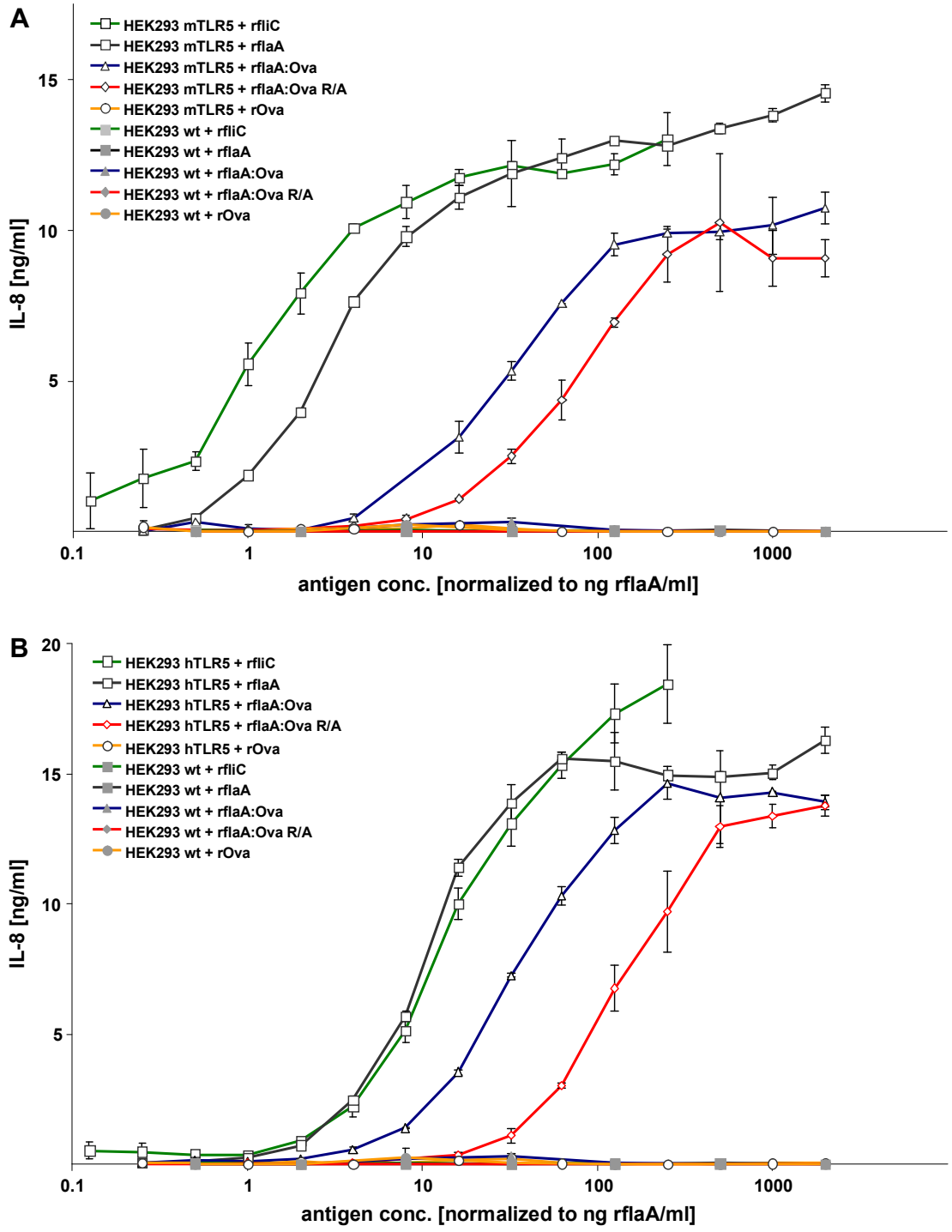


Figure 12: Recombinant flagellin A and flaA:Ova fusion proteins are able to activate murine and human TLR5. HEK293 cells stably transfected with the murine (A) or the human (B) TLR5 were stimulated with recombinant flaA, rfliC, rflaA:Ova, rflaA:Ova R/A, and rOva at the indicated protein concentrations for 22 h. Supernatants were collected and checked for the production of hIL-8 by ELISA. Non TLR5 transfected HEK293 wild type cells were stimulated as controls. Results are representative data taken from one out of two independent experiments ± SD.

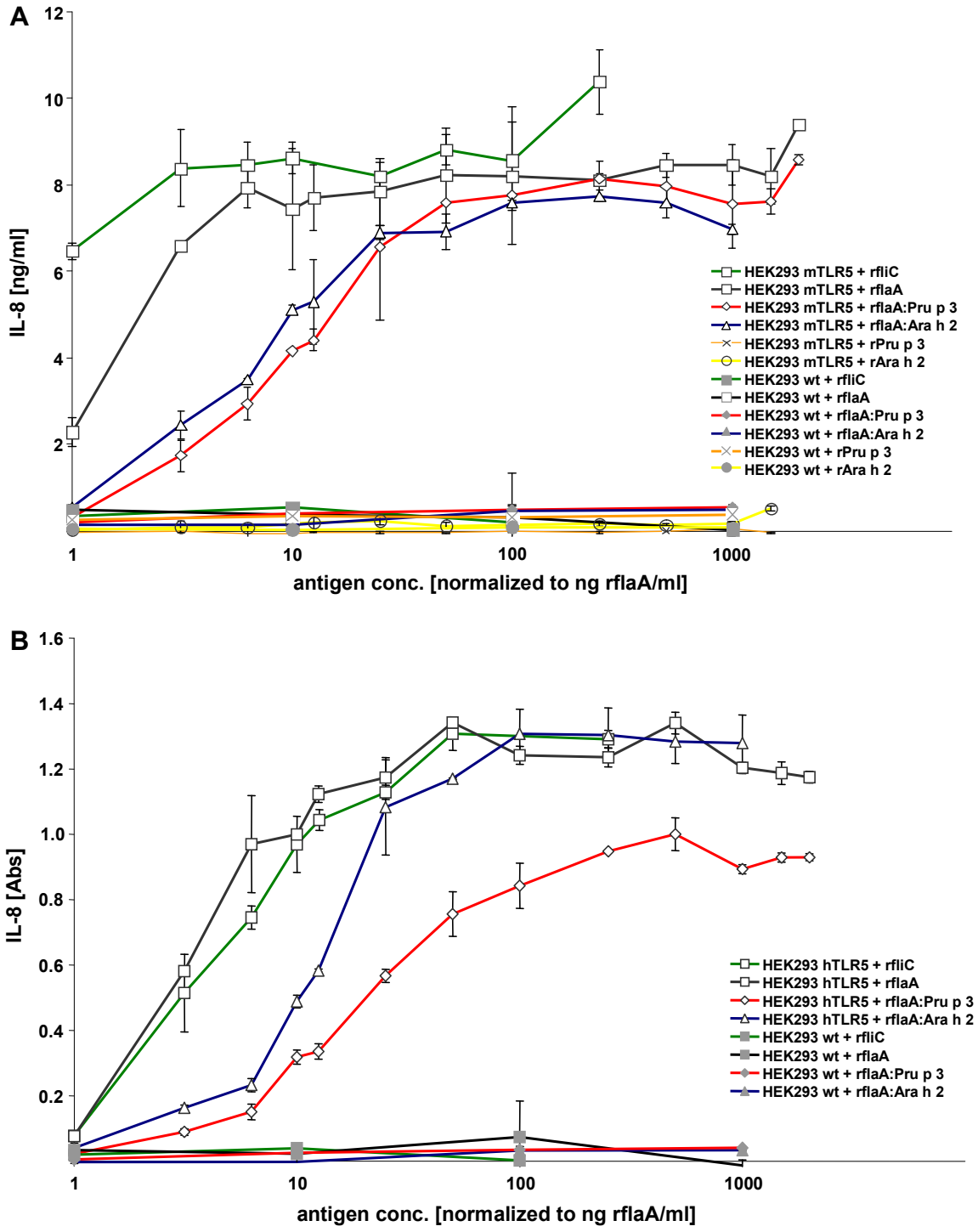


Figure 13: Recombinant flagellin A, rflaA:Pru p 3, and rflaA:Ara h 2 are able to activate murine and human TLR5. HEK293 cells stably transfected with the murine (A) or the human (B) TLR5 were stimulated with recombinant flaA, rfliC, rflaA:Pru p 3, rflaA:Ara h 2, rPru p 3, and rAra h 2 at the indicated protein concentrations for 22 h. Supernatants were collected and checked for the production of hIL-8 by ELISA. Results are expressed as absorbance (A = 450 nm) since poor staining of standard curves did not allow absolute quantification. Non TLR5 transfected HEK293 wild type cells were stimulated as controls. Results are representative data taken from one out of two independent experiments ± SD.

To test whether the aggregation observed for flagellin:allergen fusion proteins interfered with binding of the flagellin part to TLR5, rflaA and the three fusion proteins were reduced and alkylated. Subsequently, the reduced and alcylated proteins were checked for their ability to induce IL-8 secretion from TLR5 transgenic HEK293 cells (Figure 14 and Figure 12).
As shown for rflaA:Ova (Figure 10) reduction and alkylation abrogated aggregation caused by intermolecular disulfide bonds for rflaA:Pru p 3 and rflaA:Ara h 2 fusion constructs (data not shown). Reduction and alkylation of rflaA, which does not contain cysteine residues did not influence binding of rflaA to mTLR5 (Figure 14). In contrast to this partial defolding of rflaA:Ova further reduced binding to mTLR5 by factor 3 (Figure 12), whereas for rflaA:Pru p 3 and rflaA:Ara h 2 fusion proteins reduction and alkylation resulted in mTLR5 activation comparable to equimolar amounts of rflaA alone (Figure 14). Similar results were obtained when stimulating HEK293 cells expressing the human TLR5 (Figure 12, data not shown).

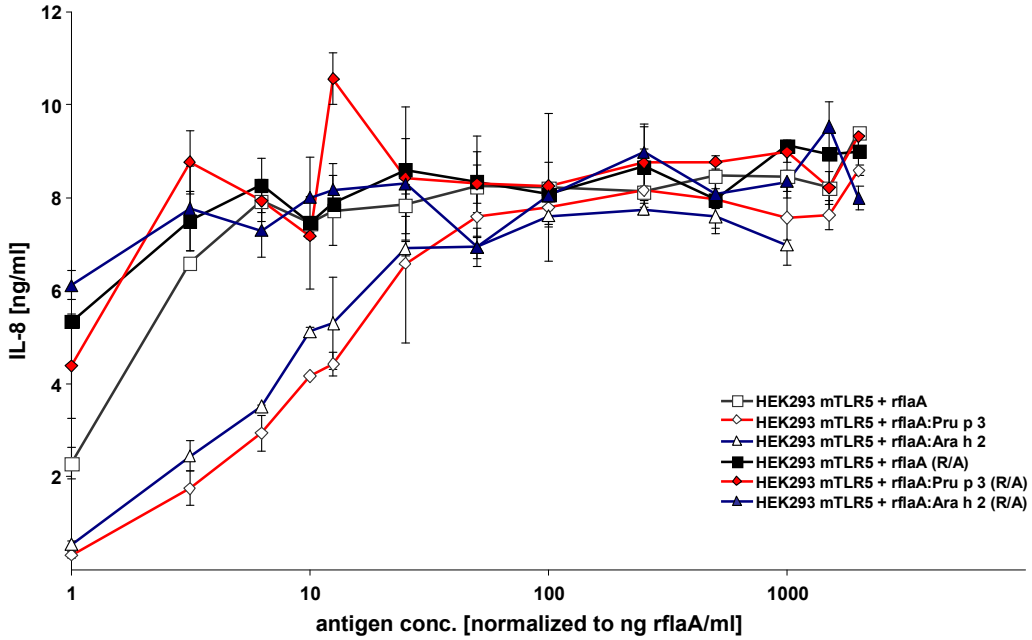


Figure 14: Reduction and alkylation of flagellin A does not influence binding to TLR5, whereas for rflaA:Pru p 3 and rflaA:Ara h 2 fusion proteins TLR5 binding is increased. HEK293 cells stably transfected with the murine TLR5 were stimulated with recombinant flaA, rflaA:Pru p 3, and rflaA:Ara h 2 either untreated or reduced and alkylated (R/A) at the indicated protein concentrations for 22 h; supernatants were collected and checked for the production of hIL-8 by ELISA. Results are representative data taken from one out of two independent experiments ± SD.

The stability of rflaA and the flaA:allergen fusion constructs was tested by boiling (for 1 h at 95°C) and proteolytic digestion. Surprisingly, even after 1 h boiling at 95°C, rflaA and the all the flagellin:allergen fusion constructs still showed approximately one third of the TLR5 activation capacity observed for the untreated proteins. As expected, proteolytic digestion with proteinase K completely abrogated IL-8 secretion (data not shown).

These findings demonstrate that the observed aggregations are likely to sterically hinder flagellin binding to TLR5. Whereas for the smaller fusion proteins (rflaA:Pru p 3 and rflaA:Ara h 2) this sterical hinderance can be abrogated by partially defolding the allergen part of the fusion protein. For fusion constructs with a higher molecular weight such as rflaA:Ova the unfolded ovalbumin part (which has a higher molcular weight than the flagellin molecule itself) may still hamper flagellin binding to its receptor.

4.2 Immune modulating properties of rflaA and rflaA fusion proteins *in vitro*

4.2.1 Generation and characterization of murine DC subsets

In order to test the properties of *L.m.* rflaA and flagellin:allergen fusion proteins to modulate innate immune responses murine DC subsets were generated (see 3.2.3). Differentiation of myeloid DC (mDC) and plasmacytoid DC (pDC) subsets from mouse bone marrow was performed according to the protocol by Waibler et al. (178). To test for homogenicity DC preparations were stained for the surface markers CD11b, CD11c and B220 and investigated by flow cytometry.

Figure 15 shows a representative FACS-staining of *in vitro* differentiated murine myeloid dendritic cells (mDC). This DC subset is characterized by the simultaneous co-expression of CD11b and CD11c, without expression of B220. For identification of mDC in a first gating step differentiated BMDC were gated for typical size and granularity, subsequently $B220^-$ but $CD11c^+$ cells were selected and checked for co-expression of CD11b. In further steps expression of target molecules (e.g. TLR5, see Figure 15) on the cell surface of $CD11b^+CD11c^+B220^-$ was analyzed. The depicted preparation shows only a small fraction of $B220^+$ cells, whereas the majority of $CD11c^+$ cells were shown to be also $CD11b^+$ and therefore represent the target cell population (Figure 15). The purity of *in vitro* differentiated mDC was depending on the preparation approximately 70 to 80% (of to all living cells). This value met the quality standards established in Waibler et al. (178).

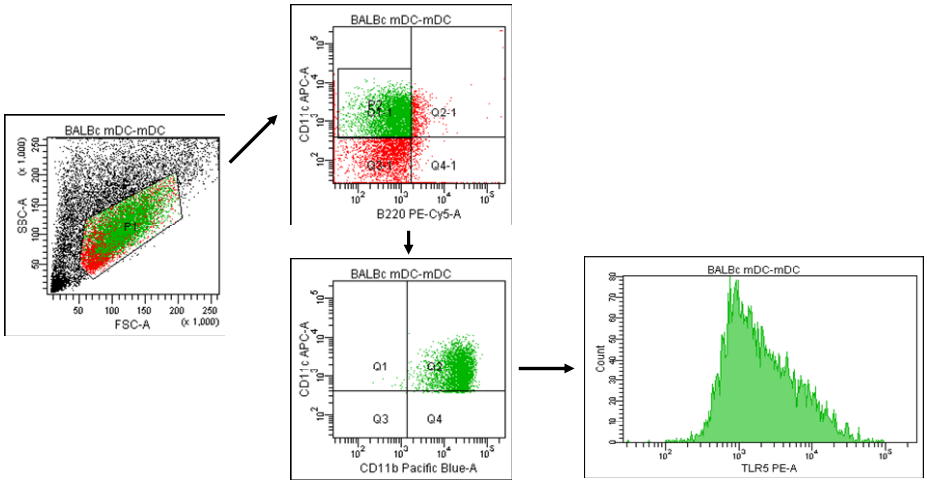


Figure 15: Quality assessment of *in vitro* differentiated mDC. Unstained samples were used to determine positive staining thresholds, putative mDC were gated for typical size and granularity. Subsequently, B220 negative but CD11c positive were gated and checked for co-expression of CD11b. Expression of target molecules (e.g. TLR5) on the cell surface of $CD11b^+CD11c^+B220^-$ was analyzed.

In contrast to mDC murine pDC are characterized by the co-expression of B220 and CD11c but do not express CD11b. For identifying the much smaller pDC subset, in a first gating step putative pDC were gated for typical size and granularity. Subsequently, $B220^{+}CD11c^{+}$ double positive cells were selected and checked for the absence of CD11b expression. Finally, the expression of target molecules on the cell surface of $CD11b^{-}CD11c^{+}B220^{+}$ pDC was analyzed (Figure 16). In comparison to mDC the purity of pDC in differentiated BMDC cultures was calculated to range from 10 to 20% (Figure 16). Moreover, absolute pDC numbers obtained after culture were reproducibly by factor 2 lower although the double amount of bone marrow cells were used for their differentiation.

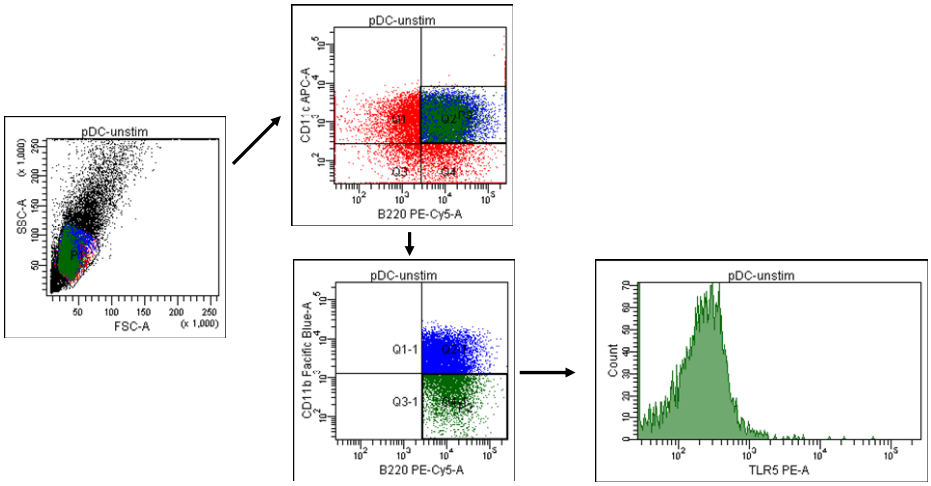


Figure 16: Quality assessment of *in vitro* differentiated pDC. Unstained samples were used to determine positive staining thresholds, putative pDC were gated for typical size and granularity. Subsequently, $B220^{+}CD11c^{+}$ cells were gated and checked for the absence of CD11b expression. Finally, the expression of target molecules (e.g. TLR5) on the cell surface of $CD11b^{-}CD11c^{+}B220^{+}$ was analyzed.

In order to exclude T and B cell contaminations in mDC and pDC preparations these were tested for CD3 positive T cells and CD19 positive B cells by flow cytometry (see 3.2.5). Hereby, mDC showed a high degree of autofluorescence due to the uptake of e.g. phenol red from cell culture media. The analysis of three independent mDC- (Figure 17) and pDC-preparations (data not shown) did not lead to the detection of either T or B cells. Therefore, a contamination with these cells could be excluded.

Overall, the quality of the investigated mDC and pDC preparations met the expected quality requirements, allowing the usage of these preparations for further experiments.

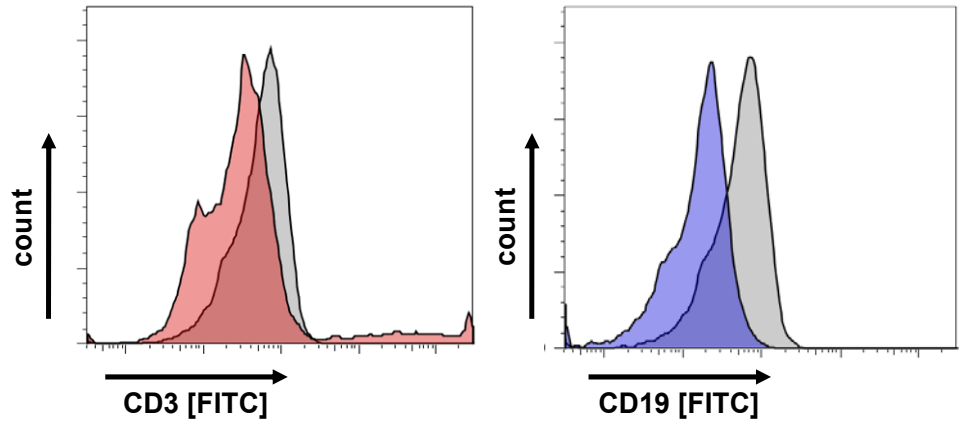


Figure 17: mDC preparations do not contain B and T cells. mDC were differentiated *in vitro* from BALB/c bone marrow for 8 days in the presence of GM-CSF and stained for CD3 and CD19. Grey: unstained cells, red: αCD3, blue: αCD19. Results were obtained gating on all living cells and are representative data taken from one out of three independent experiments.

4.2.2 rflaA:Ova potently activates dendritic cells

In order to allow a comprehensive characterization of the potential immune modulating properties of rflaA and rflaA:allergen fusion proteins in the performed *in vitro* assays rflaA and rflaA:Ova were used as model proteins. This decision was further encouraged by the commercial availability of many tools such as e.g. transgenic mice with Ova-specifc T cell receptors (OT-II, DO11.10), Ova-specific antibodies, or Ova being commercially available in high amounts.

In a first set of experiments rflaA and rflaA:Ova were tested for their ability to activate murine BALB/c-derived mDC ($CD11b^+CD11c^+B220^-$) and pDC ($CD11b^-CD11c^+B220^+$) subsets. Therefore, DC activation and cytokine secretion were investigated upon stimulation with equimolar amounts of rOva, rflaA, the mixture of rflaA and rOva, and rflaA:Ova fusion protein (either non-modified or reduced and alkylated). LPS was used as positive control. After 24 h of stimulation mDC and pDC were analyzed for surface expression of MHC I/II (data not shown), and co-stimulatory molecules CD40, CD69, CD80, and CD86 by flow cytometry (Figure 18). Upon stimulation with the fusion proteins all investigated activation markers were up-regulated on mDC. Up-regulation was strongest for CD69, intermediate for CD40 and CD86, and less pronounced for CD80 (Figure 18 and Figure 19). For all activation makers a clear up-regulation upon stimulation with LPS was detected, whereas rOva did not influence the activation status of mDC. Stimulation with rflaA alone in the used concentration did induce slight up-regulations of CD86 and CD40 in an mDC subset, whereas CD80 and CD69 expression remained unaffected. Moreover, a mixture of rflaA with rOva did not alter the flagellin-induced expression of activation markers. In contrast to this, the expression of

CD40, CD69, CD80, and CD86 upon stimulation with rflaA:Ova was strongly increased and comparable to levels observed after stimulation with LPS. In comparison to the non-modified fusion protein, rflaA:Ova (R/A) showed a similar, but slightly weaker ability to activate mDC. Furthermore, expression of MHC I and MHC II molecules on mDC was unaffected upon stimulation with the different constructs (data not shown). Similar results were obtained using mDC preparations differentiated from C57BL/6 mice (see 4.2.13).

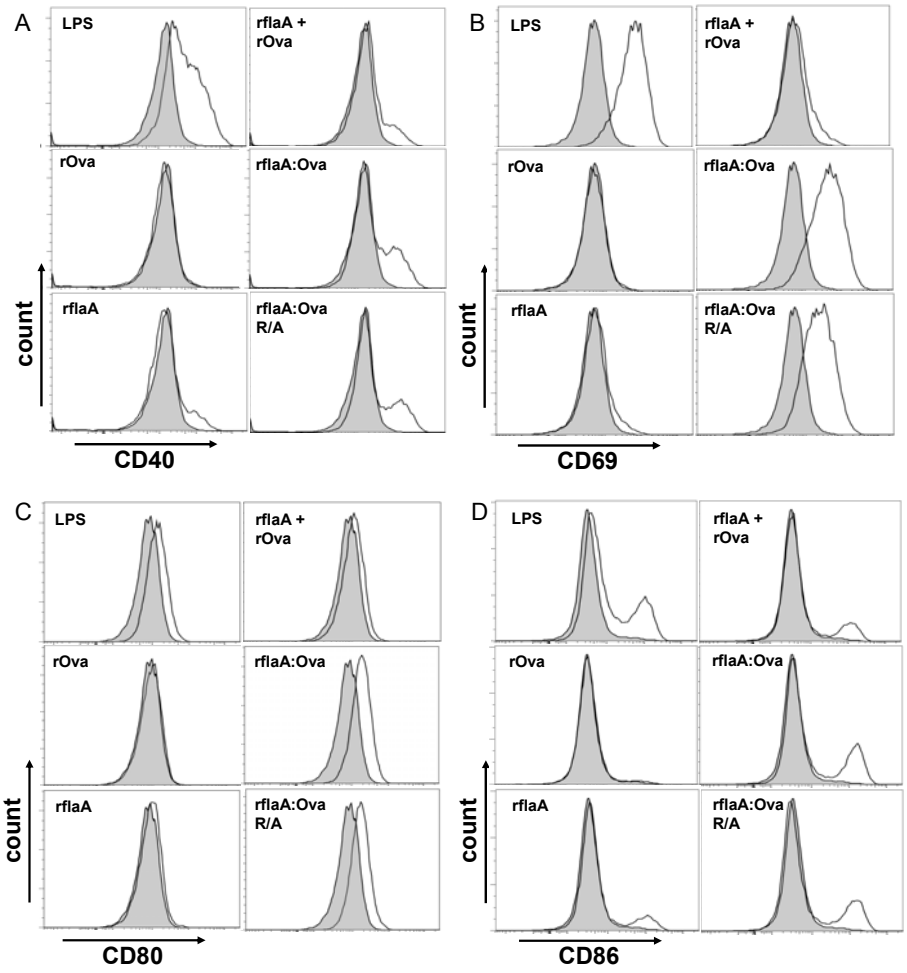


Figure 18: rflaA:Ova fusion proteins strongly activate *in vitro* generated mDC. BALB/c mDC were stimulated with LPS (10 µg/ml) and equimolar amounts of rOva (10 µg/ml), rflaA (6.9 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), rflaA:Ova (16.9 µg/ml), and rflaA:Ova R/A (16.9 µg/ml) for 24 h, gated on $CD11b^{+}CD11c^{+}B220^{-}$ mDC, and analyzed for CD40 (A), CD69 (B), CD80 (C), and CD86 (D) expression. Grey filled curves: unstimulated, black lines: stimulated as indicated. Results are representative data taken from one out of two independent experiments.

In analogous experiments using BALB/c pDC, an up-regulation of CD40 and CD69 was detected upon stimulation with either rflaA, rflaA plus rOva or rflaA:Ova but no change in CD80 and CD86 expression (Figure 19). Hereby, the observed upregulation of CD69 was stronger for rflaA:Ova than for the equimolar amount of either rflaA alone or the mixture of rflaA plus rOva. On BALB/c pDC MHC II expression was slightly upregulated only upon stimulation with rflaA:Ova (data not shown). MHC I expression on stimulated pDC was not determined. Due to limitations in protein availablity pDC were not stimulated with rflaA:Ova R/A. Moreover, due to the low activation and cytokine secretion of BALB/c derived pDC upon stimulation with the different constructs flow cytrometric analysis were not performed with C57BL/6 pDC.

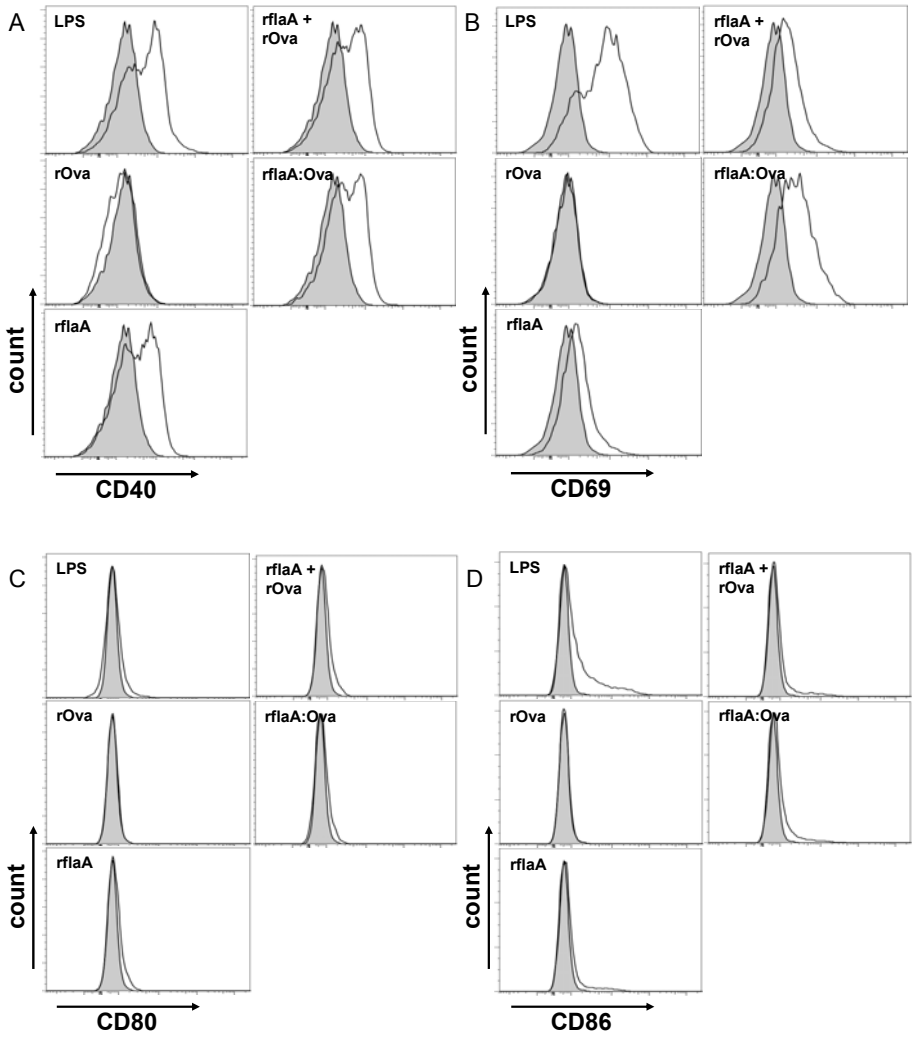


Figure 19: rflaA:Ova fusion proteins activate *in vitro* differentiated pDC. BALB/c pDC were stimulated with LPS (10 µg/ml) and equimolar amounts rOva (10 µg/ml), rflaA (6.9 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), and rflaA:Ova (16.9 µg/ml) for 24 h, gated on $CD11b^{-}CD11c^{+}B220^{+}$ pDC, and analyzed for CD40 (A), CD69 (B), CD80 (C), and CD86 (D) expression. Grey filled curves: unstimulated, black lines: stimulated as indicated. Results are representative data taken from one out of two independent experiments.

To further study the activation of BALB/c DC subsets, supernatants of cells stimulated with the different constructs were analyzed for their cytokine profiles (Figure 20 and Figure 21). Statistical analysis was performed according to chapter 3.4 using a mixed linear model with mixed factor treatment group and a random factor assay (see 3.4). Recombinant flaA:Ova (non-modified and R/A) induced the production of IL-1ß, IL-6 as well as IL-10 by mDC (Figure 20), whereas only minor amounts of the pro-inflammatory cytokine IL-12 could be detected (approx. 90 pg/ml, Figure 21). In all experiments the rflaA:Ova fusion protein induced significantly higher amounts of IL-1β (5-fold), IL-6 (>17-fold), and IL-10 (>140-fold) than both proteins provided alone or as an equimolar mixture. Recombinant flaA:Ova (R/A) induced even higher amounts of IL-6 than the non-modified fusion protein, whereas levels of IL-1ß and IL-10 were comparable for both proteins. In the used concentrations neither rflaA nor rOva alone or both provided as a mixture did induce significant amounts of IL-10 and IL-6. According to the available literature rflaA stimulation induced low levels of IL-1ß secretion. As expected, LPS used as positive control triggered IL-1β, IL-6, IL-10, and IL-12 secretion from mDC (Figure 20). Similar results were obtained using mDC generated from C57/BL6 mice (data not shown, see 4.2.12).

In supernatants of analogously stimulated pDC (either derived from BALB/c or C57BL/6 mice) similar levels of IL-10 but almost no IL-6 were detected (data not shown). Since the used DC preparations were characterized by a purity of 70 to 80% for mDC and only 10 to 20% for pDC, intracellular cytokine stainings (see 4.2.1) had to be performed in order to verify mDC and pDC as the source of the secreted cytokines.

In summary the rflaA:Ova fusion protein was shown to induce superior DC activation compared to equimolar amounts of flaA alone or the mixture of rflaA and Ova. Here mDC were shown to react stronger upon stimulation with rflaA:Ova than pDC.

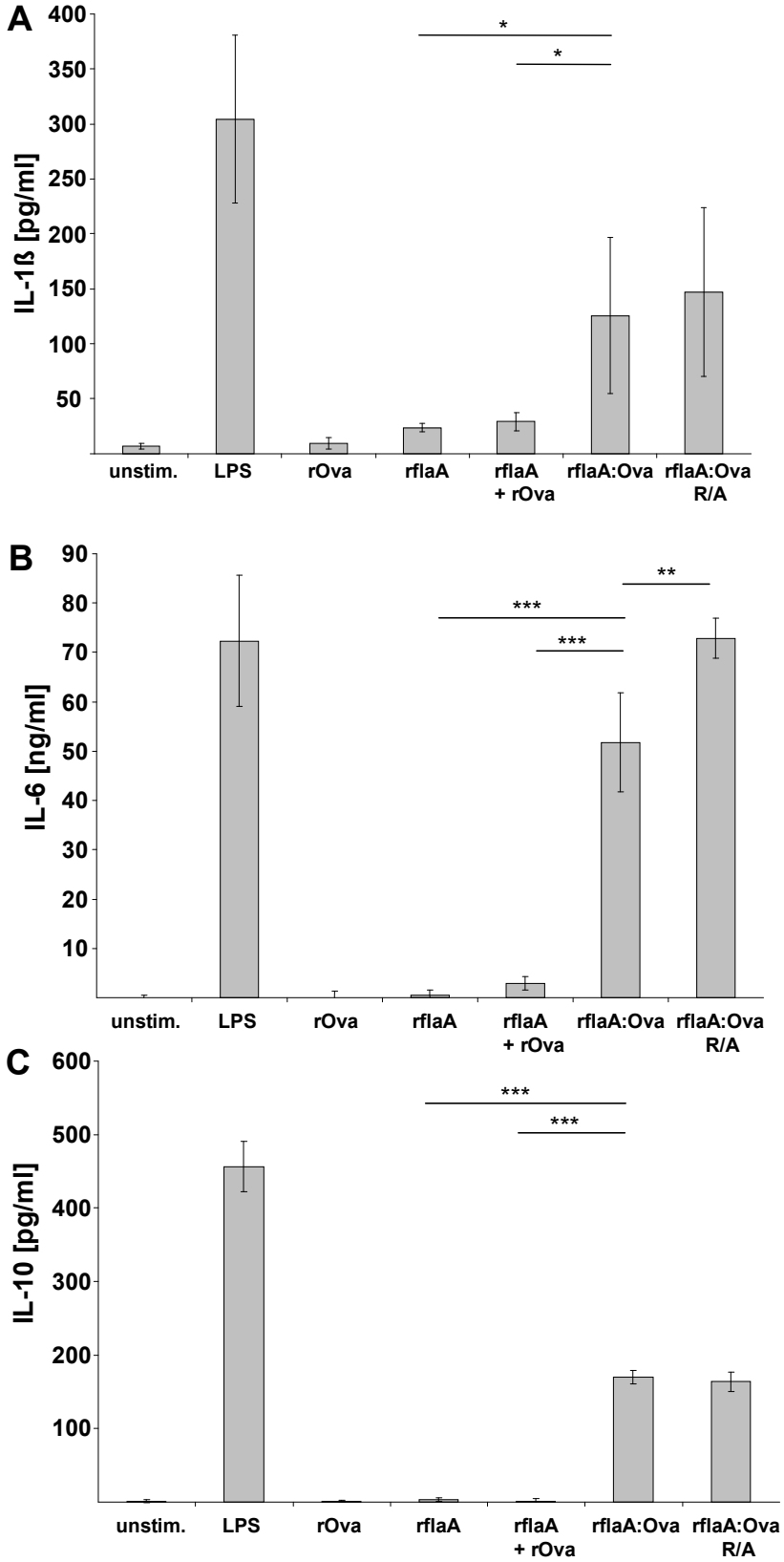


Figure 20: rflaA:Ova induces IL-1β, IL-6, and IL-10 secretion from mDC. BALB/c mDC were stimulated with LPS (10 µg/ml) and equimolar amounts of rOva (10 µg/ml), rflaA (6.9 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), rflaA:Ova (16.9 µg/ml, non-modifed and R/A). Levels of IL-1β (A) and IL-6 (B) were detected after 24 h, levels of IL-10 (C) after 72 h of stimulation. Results are mean values ± SD of three independent experiments.

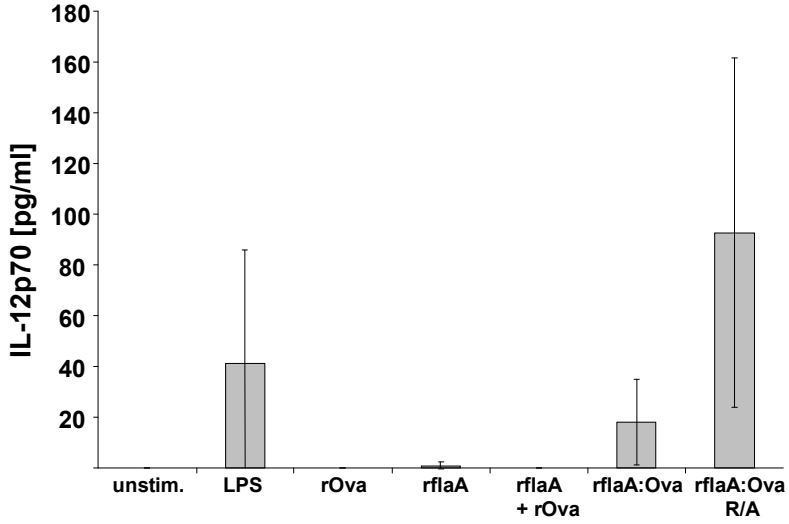


Figure 21: rflaA:Ova and rflaA do not induce significant IL-12 production from mDC. BALB/c mDC were stimulated with LPS (10 µg/ml) and equimolar amounts of rOva (10 µg/ml), rflaA (6.9 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), or rflaA:Ova (16.9 µg/ml, non-modifed and R/A). Levels of IL-12p70 were detected after 24 h of stimulation. Results are mean values ± SD of three independent experiments.

4.2.3 Intracellular cytokine staining confirms mDC as the source of the secreted cytokines

To substantiate the ELISA results and to check whether IL-6 and IL-10 secretion observed in GM-CSF and Flt-3L cultures were produced by mDC and pDC respectively, intracellular cytokine stainings (ICS) for IL-10 were performed in stimulated mDC and pDC (Figure 22). In accordance with the results obtained by ELISA, stimulation with both rflaA:Ova proteins (non-modified and R/A) induced IL-6 and IL-10 production in $CD11b^{+}CD11c^{+}B220^{-}$ mDC, whereas rflaA and rOva applied alone or as a mixture did not induce cytokine secretion (Figure 22 and Figure 23). As expected, LPS induced the production of IL-6 and IL-10, whereas no change in cytokine production was observed upon co-administration of LPS with rOva (data not shown).

In contrast to this, in Flt-3L cultures IL-10 secretion determined by ELISA upon stimulation with the fusion protein could not be assigned to $CD11b^{-}CD11c^{+}B220^{+}$ pDC by ICS (Figure 22) but was likely caused by $CD11b^{+}CD11c^{+}$ conventional DC (cDC) or $CD11b^{+}CD11c^{+}B220^{-}$ mDC contained within the Flt-3L cultures. Moreover, since neither BALB/c nor C57BL/6 derived pDC did produce significant amounts of IL-6 upon stimulation (data not shown) ICS for IL-6 was not performed in pDC.

In summary, ICS confirmed that rflaA:Ova fusion proteins induced high amounts of IL-6 and IL-10 from mDC but not from pDC. Additionally, in contrast to mDC pDC showed weaker expression of activation markers and no cytokine secretion upon stimulation with the different

constructs: Therefore, mDC are very likely the more important DC subset to study the effects of flagellin and flagellin:allergen fusion proteins. Because of this reason the following experiments focused on the immune modulating properties of the different constructs using mDC as antigen presenting cells.

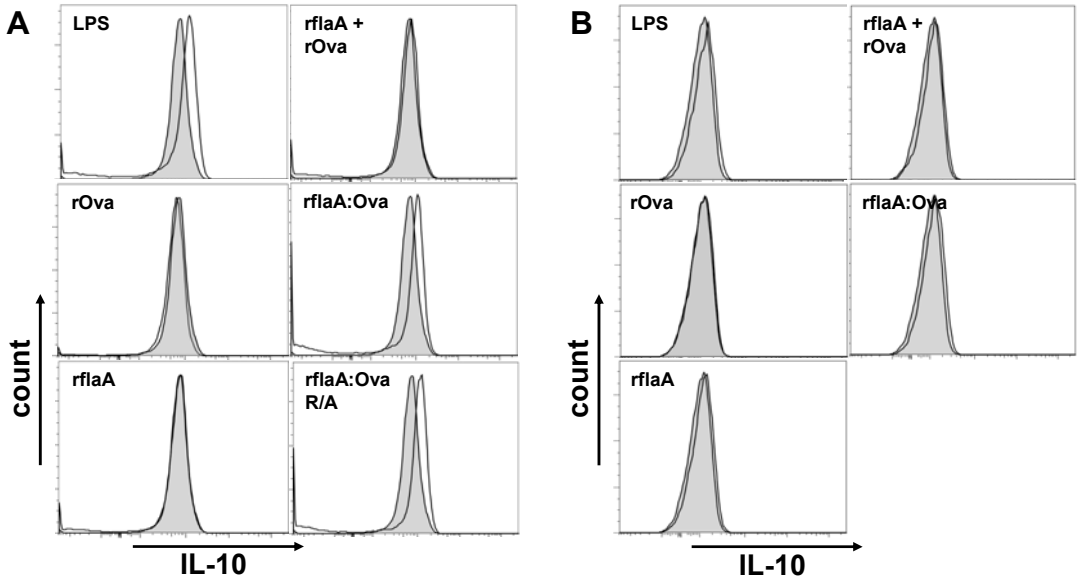


Figure 22: Intracellular cytokine staining confirms mDC but not pDC as producers of IL-10 upon stimulation with rflaA:Ova. BALB/c mDC (A) or pDC (B) were stimulated with LPS (10 µg/ml) and equimolar amounts rOva (10 µg/ml), rflaA (6.9 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), and rflaA:Ova (16.9 µg/ml) for 24 h. mDC were gated on $CD11c^{+}CD11b^{+}B220^{-}$, pDC on $CD11c^{+}CD11b^{-}B220^{+}$ cells, and intracellular cytokine staining of IL-10 was investigated. Results are representative data taken from one out of two independent experiments.

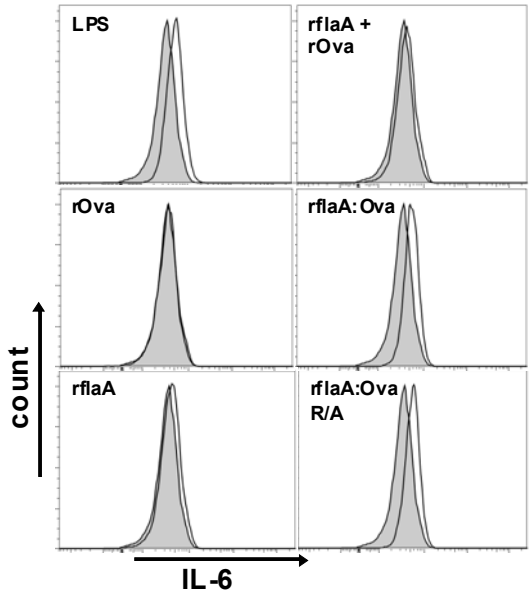


Figure 23: mDC produce IL-6 upon stimulation with rflaA:Ova. BALB/c mDC were stimulated with LPS (10 µg/ml) and equimolar amounts rOva (10 µg/ml), rflaA (6.9 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), and rflaA:Ova (16.9 µg/ml) for 24 h. mDC were gated on $CD11c^{+}CD11b^{+}B220^{-}$, and intracellular cytokine staining of IL-6 was assessed. Results are representative data taken from one out of two independent experiments.

4.2.4 rflaA:Ova mediated cell activation and cytokine secretion is independent of LPS

In order to exclude immunologic effects of residual LPS contaminations on the observed DC responses, the sensitivity of BALB/c mDC towards LPS was determined. For this purpose mDC were stimulated for 24 h with LPS amounts ranging from 1 pg/ml to 10 µg/ml. Subsequently, supernatants were checked for the production of IL-6 and IL-10 by ELISA. Hereby, an initial IL-6 induction was observed when applying more than 100 pg/ml LPS, whereas IL-10 production was first detectable using concentrations of at least 0.7 ng/ml LPS (Figure 24). The rflaA:Ova amount used for mDC stimulation contained approximately 49 pg LPS per 16.9 µg protein, which is well below the determined threshold values (100 pg/ml for IL-6 and 0.7 ng/ml for IL-10). Therefore residual LPS contained in the used rflaA:Ova preparation does not contribute to cytokine secretion.

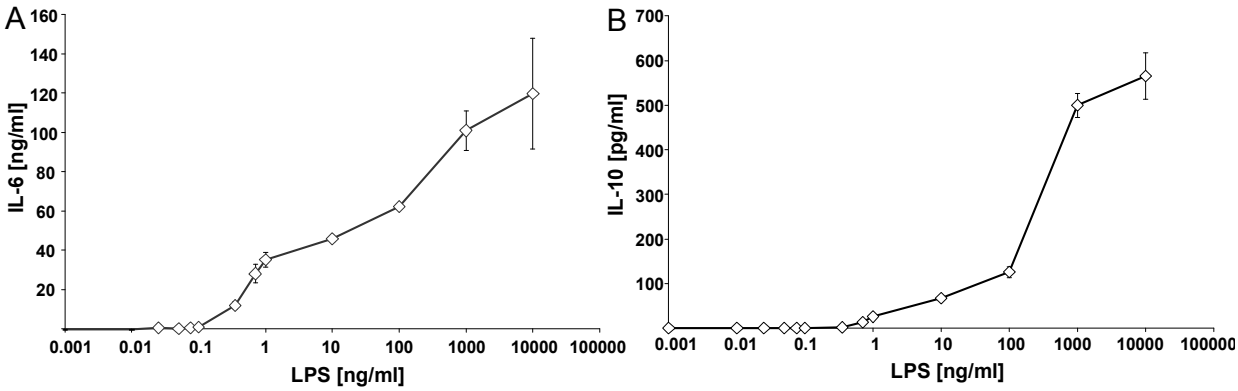


Figure 24: LPS and rflaA-induced cytokine secretion. BALB/c mDC were stimulated with LPS and levels of IL-6 (A) and IL-10 (B) in the culture supernatant were determined after 24 h by ELISA. Results for IL-6 are mean values ± SD of two independent experiments, whereas results for IL-10 were obtained from one experiment ± SD.

In line with these results, stimulation of mDC with LPS amounts corresponding to the ones contained in the used rflaA:Ova preparation (e.g. 49 pg LPS per 16.9 µg protein) did not result in detectable IL-10 secretion (Figure 25). Moreover, proteolytic digestion of rflaA:Ova using proteinase K, which degrades the protein but not the LPS, abolished IL-10 secretion from mDC (Figure 25), demonstrating the observed cytokine secretion to be exclusively mediated by the protein and not by residual LPS-contaminations.

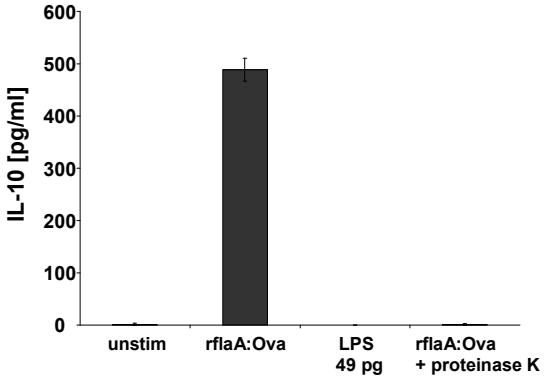


Figure 25: rflaA:Ova-induced IL-10 secretion is independent of LPS. BALB/c mDC were stimulated with rflaA:Ova (16.9 µg/ml), the corresponding amount of LPS contained in 16.9 µg rflaA:Ova (49 pg) and proteinase K digested rflaA:Ova (16.9 µg/ml) for 24 h. IL-10 secretion was analyzed by ELISA. Results are mean of 2 technical replicates ± SD.

4.2.5 mDC are activated by high doses of rflaA

Remarkably, the applied concentration of rflaA induced slight cell activation, but only negligible secretion of IL-1β, IL-6, and IL-10 (see Figure 18 and Figure 20). In order to investigate whether increasing concentrations of rflaA itself were able to induce cytokine production from BALB/c mDC, cells were stimulated with equimolar amounts of rflaA and rflaA:Ova, equivalent to 0.25 to 64 µg/ml flagellin, and analyzed for IL-6 and IL-10 production. Whereas stimulation with the fusion protein in the lowest concentration (corresponding to 0.25 µg/ml rflaA) readily induced IL-6 (approx. 20 ng/ml) and IL-10 (approx. 5 pg/ml) secretion, for rflaA a detectable IL-6 and IL-10 production was first observed upon stimulation with 32 µg/ml flaA (Figure 26). Stimulation with corresponding amounts of LPS could show these effects to be independent of residual LPS contaminations.

These experiments demonstrate that rflaA in a concentration of at least 32 µg/ml was able to induce cytokine secretion in the applied *in vitro* model system, although in direct comparison to rflaA:Ova approximately 100-fold higher protein concentrations had to be applied.

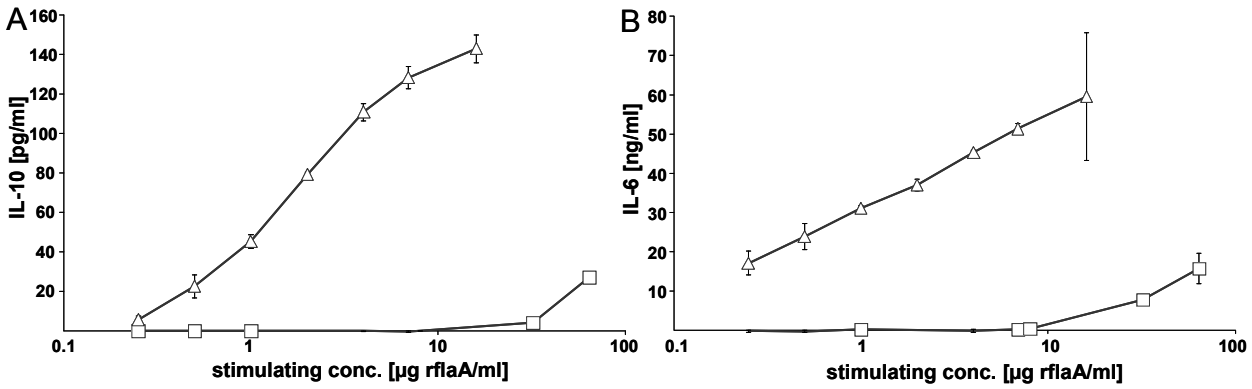


Figure 26: High concentrations of rflaA induce cytokine secretion from mDC. BALB/c mDC were stimulated with rflaA (squares), and rflaA:Ova (triangles) for 24 h. Levels of IL-10 (A) and IL-6 (B) in the culture supernatant were determined by ELISA. rflaA- and rflaA:Ova-induced cytokine secretion were corrected for LPS-induced cytokine secretion by stimulating mDC with corresponding amounts of LPS and substracting the LPS-obtained cytokine values from the values measured by ELISA. Results for IL-6 are mean values ± SD of two independent experiments, whereas results for IL-10 were obtained from one experiment ± SD.

4.2.6 rflaA:Ova rapidly incduces long lasting cytokine secretion

To further characterize rflaA:Ova-induced cytokine secretion and determine the optimal endpoint for further experiments the time course of IL-6 and IL-10 secretion by BALB/c mDC was determined (Figure 27). IL-6 secretion induced by rflaA:Ova and LPS stimulation was detected as early as 2 to 4 h post stimulation (Figure 27 A). Here, levels of IL-6 peaked at 24 h after stimulation (Figure 27) and remained unchanged up to 72 h post stimulation (data not shown). LPS- as well as rflaA:Ova-induced IL-10 secretion was first detected after 6 h of stimulation, reached its maximum after 12 h (Figure 27 B), and stayed constant until 72 h post stimulation (data not shown). In agreement with the experiments presented above (see 4.2.2), in the used concentrations neither rflaA nor rOva provided alone or as a mixture did induce cytokine secretion.

Due to these results, 24 h stimulation was applied in further experiments when stimulating mDCs alone or 72 h when performing mDC:T cell co-cultures.

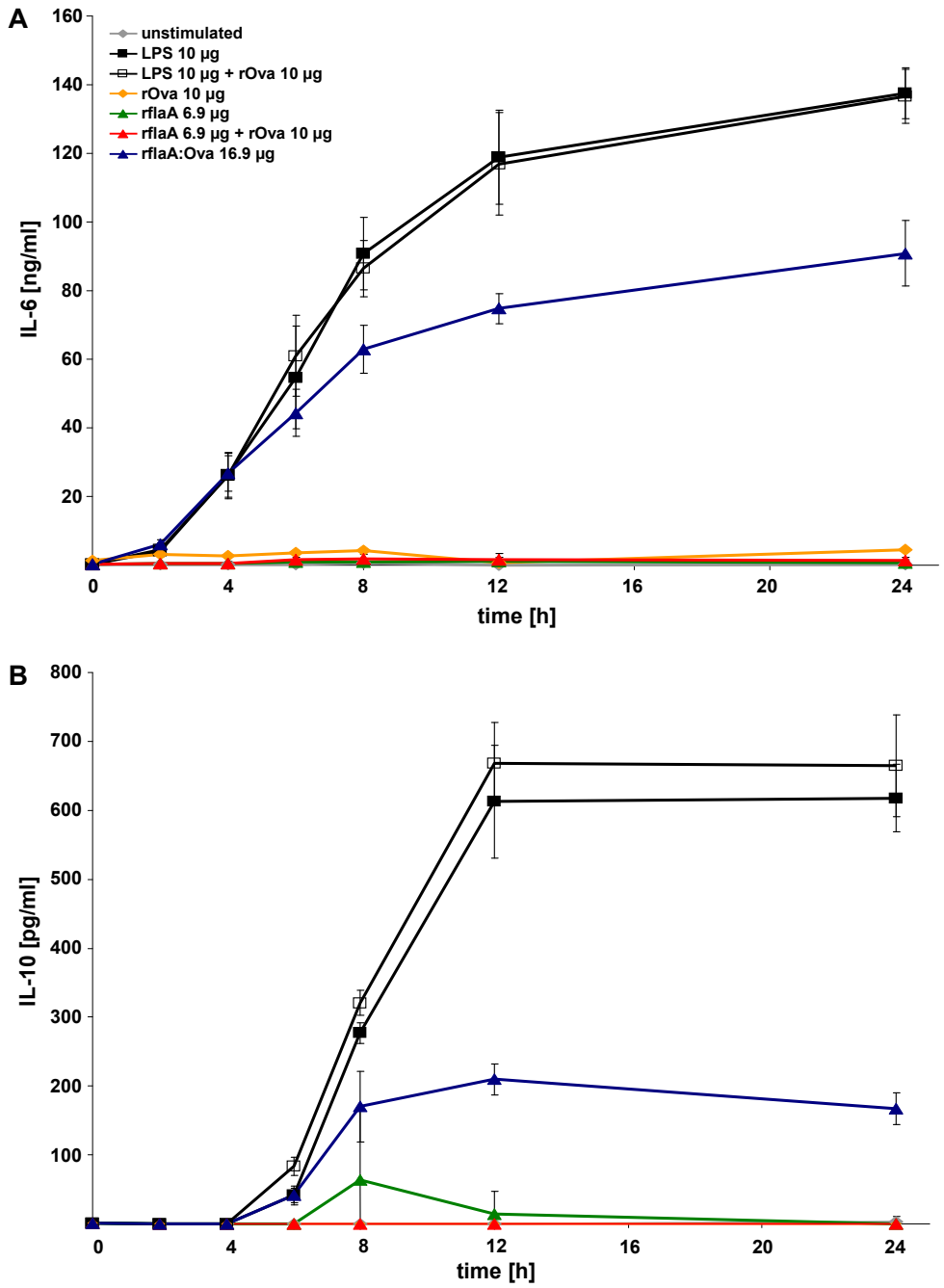


Figure 27: Time kinetics of rflaA:Ova-induced IL-6 and IL-10 production. BALB/c mDCs were stimulated with the indicated protein concentrations, supernatants were taken after 2, 4, 6, 8, 12, and 24 h. Levels of IL-6 (A) and IL-10 (B) were determined by ELISA. Data are mean of 3 independent experiments ± SD for IL-6 and two independent experiments for IL-10.

4.2.7 rflaA:Ova induces a strong upregulation of TLR5

In order to explain the observed cell activation and cytokine production mDC were stimulated and analyzed for their surface expression of the flagellin receptor TLR5 (Figure 28). Whereas levels of TLR5 expression on unstimulated mDC were very low or even not detectable, stimulation with LPS and rflaA:Ova (either non-modified or R/A) induced a strong upregulation of TLR5 expression (Figure 28 A). In accordance with the results obtained for co-stimulatory molecules the partly defolded rflaA:Ova (R/A) was less effective in inducing TLR5 expression compared to the non-modified but aggregated rflaA:Ova (Figure 18). At the protein concentration used rflaA (6.9 µg/ml) failed to induce a significant TLR5 upregulation. However, when applying higher amounts of rflaA an upregulation of TLR5 was detected (Figure 29). As expected, stimulation with rOva did not induce TLR5 expression on mDC, and when rflaA and rOva were provided as a mixture, TLR5 expression did not change compared to rflaA stimulated cells.

In summary, the analysis of TLR5 expression levels on the stimulated mDC revealed that, in accordance with all results presented above, rflaA:Ova induced a strong TLR5 upregulation whereas the equimolar amount of flagellin, or the mixture of both components did not show comparable effects. Analogous experiments with *in vitro* generated BALB/c pDC did not result in detectable TLR5 expression on either unstimulated or stimulated cells (Figure 28 B), substantiating the result that mDC are the more important DC subset to study the effects of flagellin and flagellin fusion proteins.

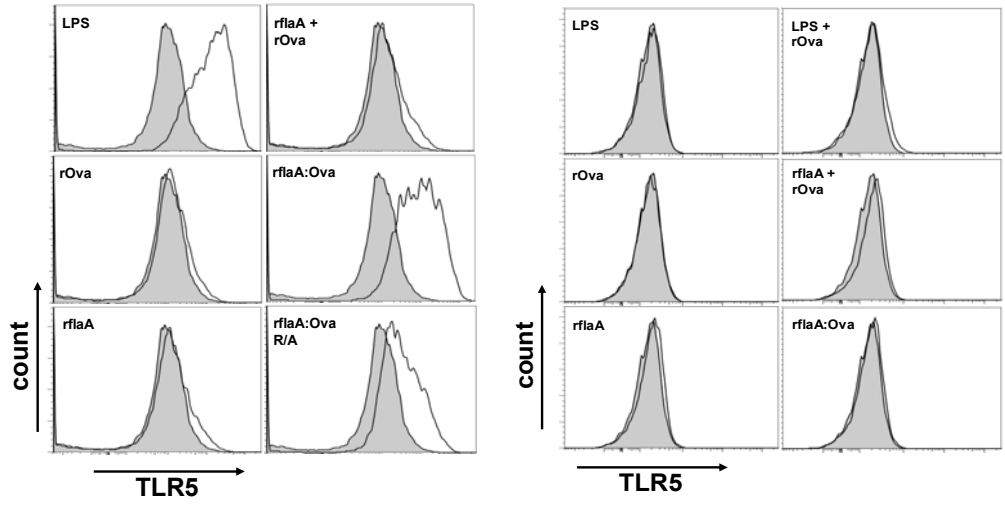


Figure 28: rflaA:Ova induces TLR5 expression on *in vitro* generated mDC but not on pDC. BALB/c mDC (A) and pDC (B) were stimulated with LPS (10 µg/ml) and equimolar amounts of rOva (10 µg/ml), rflaA (6.9 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), rflaA:Ova (16.9 µg/ml), and rflaA:Ova R/A (16.9 µg/ml) for 24 h. Cells were stained for CD11b, CD11c, B220, and TLR5. mDC were gated on $CD11b^+CD11c^+B220^-$ cell fractions, pDC were gated on $CD11b^-CD11c^+B220^+$ cell fractions, and TLR5 expression was investigated. Grey filled lines: unstimulated cells, black lines: stimulated with indicated construct. Results show representative data taken from one out of two independent experiments.

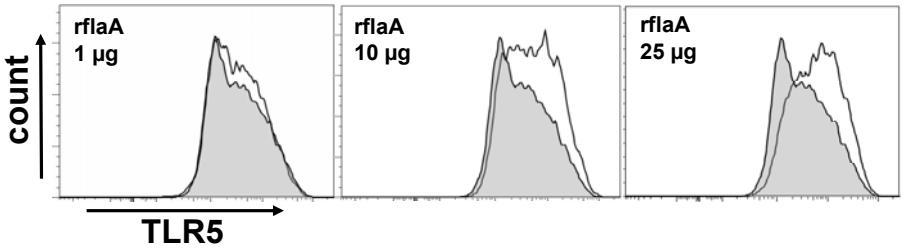


Figure 29: High concentrations of rflaA induce TLR5 upregulation on mDC. BALB/c mDC were stimulated with the indicated amounts of rflaA for 24 h, and $CD11b^{+}CD11c^{+}B220^{-}$ mDC were analyzed for TLR5 expression. Grey filled curves: unstimulated cells, black lines: stimulated as indicated. Results show representative data taken from one out of two independent experiments.

4.2.8 rflaA:Ova mediated DC activation is similar to other TLR-ligands

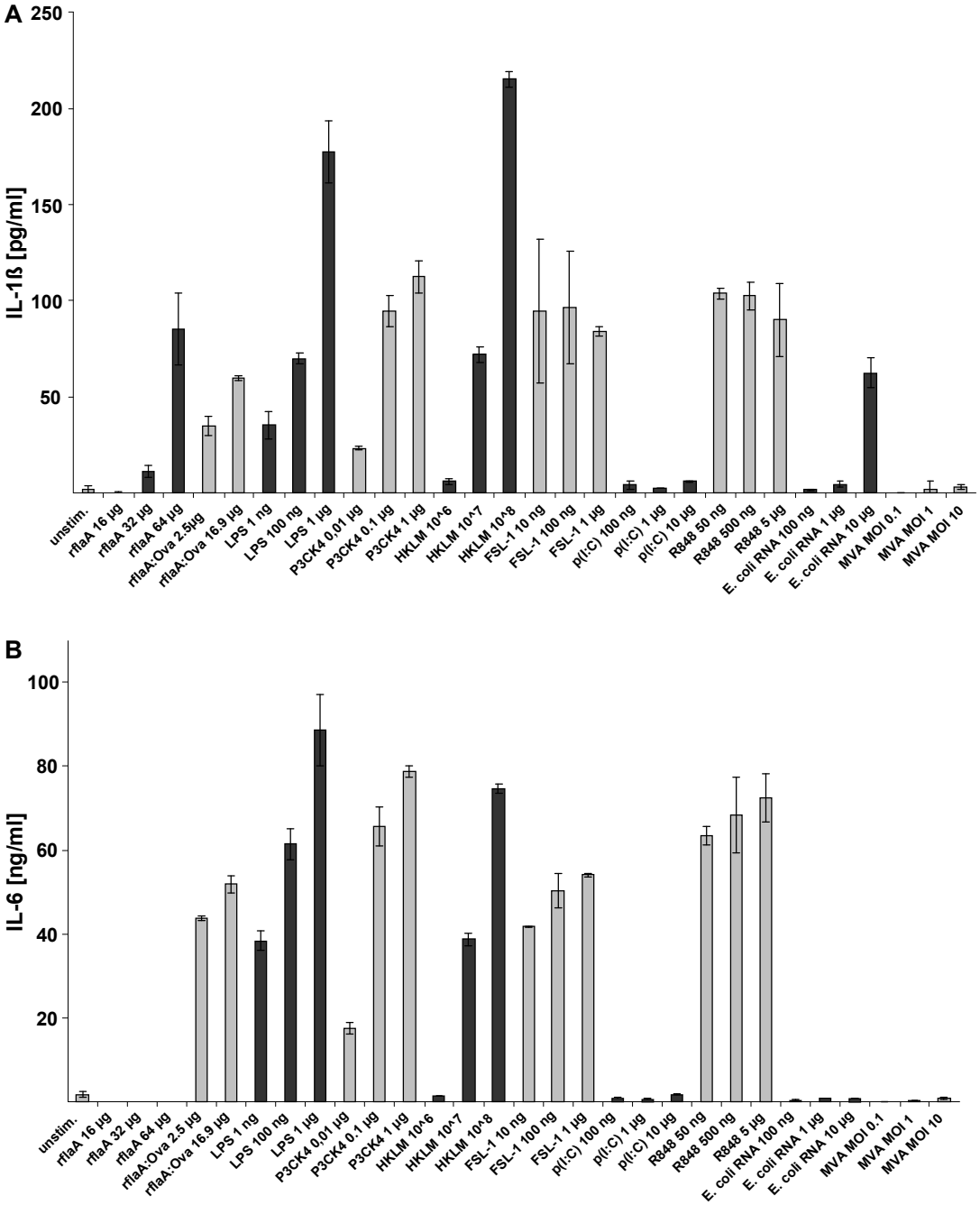
In order to further evaluate the potency of rflaA:Ova to activate murine mDC, the fusion proteins ability to activate mDC and induce cytokine secretion was directly compared to other TLR-ligands. As control for effects possibly induced by residual LPS-contaminations, stimulation of C57BL/6 mDC with the LPS amounts contained in the rflaA concentrations used (176 pg LPS in 16 µg rflaA, 352 pg LPS in 32 µg rflaA, and 704 pg LPS in 64 µg rflaA) were performed (Figure 30). For cytokine determination by ELISA (see 3.2.6) results obtained for LPS controls were subtracted from the results obtained for rflaA stimulations in order to adjust for LPS-induced cytokine secretion (Figure 30).

With the exception of the DNA-virus MVA and the TLR3-ligand pI:C all TLR-ligands induced considerable IL-1β (Figure 30 A) secretion from C57BL/6 mDC. Compared to the other TLR-ligands, the rflaA:Ova-induced IL-1β secretion of 40 to 67 pg/ml was in the medium range, but stronger than the IL-1β secretion induced by flagellin A stimulation alone (due to the experimental results presented under 4.2.2 much lower protein amounts were used for stimulation with rflaA:Ova than for rflaA). In comparison to 10^7 HKLM 16.9 µg/ml rflaA:Ova induced comparable IL-1ß secretion (Figure 30 B).

When investigating the secretion of IL-6, apart from rflaA, polyI:C, *E. coli* RNA, and MVA, all investigated TLR-ligands induced a dose-dependent secretion of IL-6 ranging from approximately 20 ng/ml at the lower concentrations to 90 ng/ml at the highest concentrations used (Figure 30 B). In this experimental setting IL-6 levels induced by stimulation with rflaA:Ova were comparable to the ones induced by other TLR-ligands although relatively low amounts of rflaA:Ova were used for stimulation. Here, IL-6 secretion induced by stimulation with rflaA:Ova was equivalent to the one induced by10^7 to 10^8 HKLM.

Upon comparison of TLR-ligand-induced IL-10 secretion only TLR5-ligand rflaA:Ova, TLR4-ligand LPS, TLR2-ligand HKLM, and TLR7/8-ligand R848 did induce detectable levels of IL-10 (Figure 30 C). Hereby, the highest concentration of HKLM, resulting in nearly 3000 pg/ml IL-10 was by far the strongest inducer of IL-10 secretion. Levels of rflaA:Ova induced

IL-10 secretion lay around 400 pg/ml and were comparable to IL-10 secretion induced by 100 ng/ml LPS (400 pg/ml) and 5 µg/ml R848 (500 pg/ml, Figure 30 C).



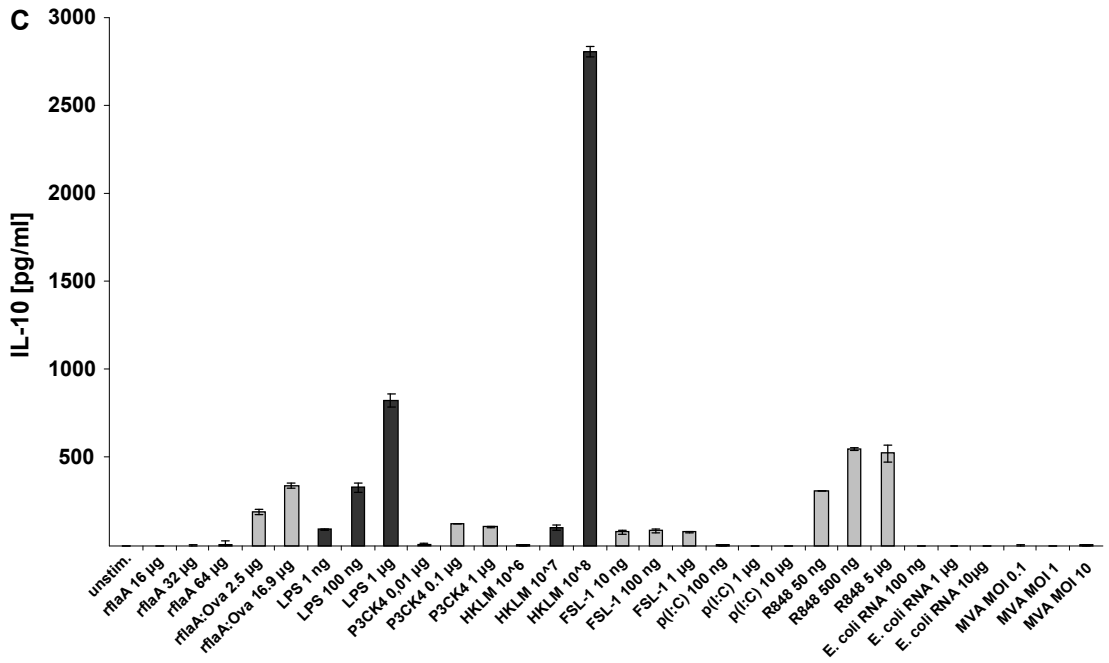


Figure 30: rflaA:Ova-induced cytokine secretion is similar to other TLR-ligands. C57BL/6 mDC were stimulated with the indicated TLR-ligands for 24 h. Supernatants were analyzed for levels of IL-1β (A), IL-6 (B), and IL-10 (C). Data are representative results ± SD taken from one out of three independent experiments.

Cell activation measured by upregulation of CD40 was only observed after stimulation with rflaA (> 64 µg), rflaA:Ova (> 2.5 µg/ml), LPS (> 100 ng/ml), and R858 (> 500 ng/ml). This upregulation was stronger for rflaA:Ova and LPS than for rflaA and R848, respectively (Figure 31 A). Moreover, the early activation marker CD69 showed a similar upregulation upon stimulation with rflaA (> 32 µg/ml), rflaA:Ova (> 2.5 µg/ml), LPS (> 700 ng/ml), and R848 (> 50 ng/ml) but was additionally dose-dependently upregulated by stimulation with polyI:C (> 1 µg/ml) and E. coli RNA (> 100 ng/ml, Figure 31 B). Stimulation of mDC with LPS amounts contained in the rflaA concentrations used showed the observed cell activation by rflaA to be at least in part independent of the LPS amounts present in the preparation used. No mDC activation was observed for Pam_3CysK_4, HKLM, and FSL-1 although these TLR-ligands induced considerable cytokine secretion (Figure 30).

In summary, these experiments demonstrated rflaA in comparison to rflaA:Ova, or LPS to be a rather weak stimulus for the investigated mDC subset. In contrast to this, rflaA:Ova-mediated mDC activation and cytokine secretion were comparable to other TLR-ligands, proving fusion proteins containing flagellin to be strong stimuli for mDC.

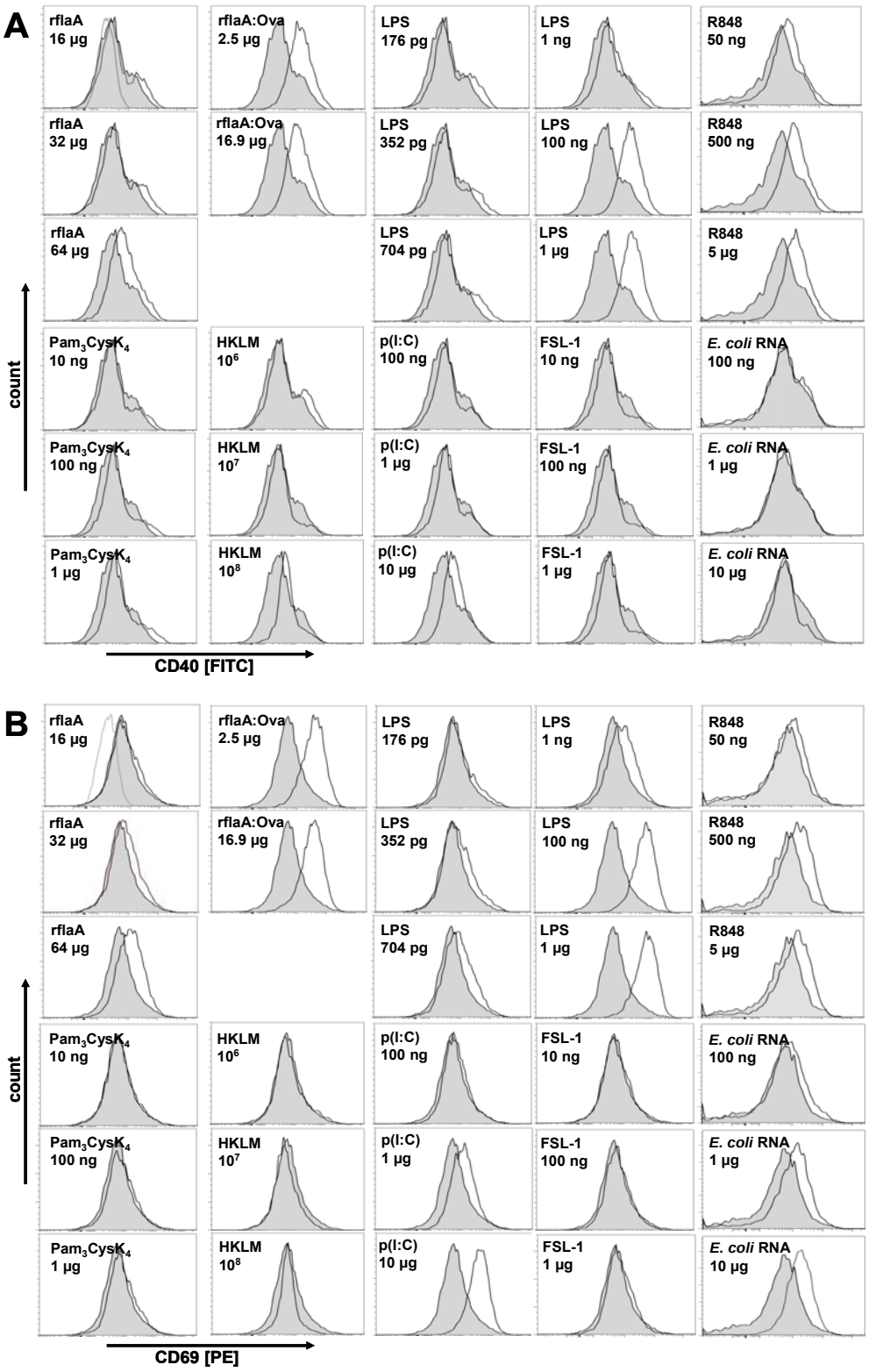


Figure 31: mDC activation capacitiy of rflaA:Ova is similar to other TLR-ligands. C57BL/6 mDC were stimulated with the indicated TLR-ligands in duplicates or triplicates for 24 h. Subsequently, cells were harvested, washed, pooled, and stained for CD11b, CD11c, B220, CD69, and CD40; gated on $CD11b^{+}CD11c^{+}B220^{-}$ mDC, and analyzed for CD40 (A) and CD69 (B) expression; grey dotted lines: unstained, grey solid lines: unstimulated, black: stimulated with indicated construct. Data are representative results taken from one out of two independent experiments. Isotype controls were performed and showed no unspecific binding.

4.2.9 rflaA:Ova represses IL-4 and IFN-γ secretion by Ova-specific T cells

To further investigate the immune modulating abilities of the fusion constructs, stimulated mDC were used as APCs in co-culture experiments with MACS purified CD4 T cells (Figure 32) isolated from DO11.10 mice. DO11.10 cells express a transgenic T cell receptor specific for Ova. T cell activation was evaluated in respect to cytokine production (IL-2, IL-4, IL-6, IL-10, IL-17A, IL-23, TGF-β, and IFN-γ), whereas mDC cultivated without T cells served as controls (Figure 33). Statistical analysis was performed according to chapter 3.4 using a mixed linear model with mixed factor treatment group and a random factor assay (see 3.4).

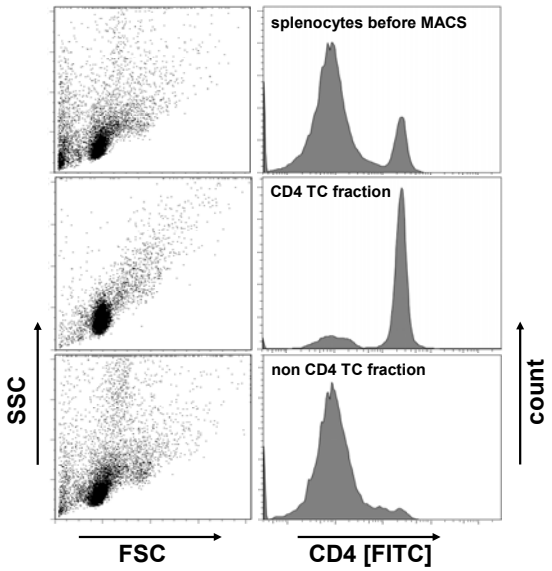


Figure 32: CD4 T cell purification by magnetic cell sorting. CD4 T cells were purified from BALB/C, DO11.10, and OT-II splenocytes using the CD4 T Cell Isolation Kit. Purity was confirmed by flow cytometry (Miltenyi Biotec, Bergisch-Gladbach).

Remarkably, rflaA:Ova-induced IL-2 secretion from CD4 T cells (2500 to 2800 pg/ml) was at least as high as the levels observed for the equimolar amount of rOva (applied alone or in a mixture with rflaA, approximately 2000 pg/ml for rOva). Upon stimulation with rflaA:Ova (R/A) similar amounts of IL-2 were induced compared to the non-modified fusion protein (Figure 33). This demonstrates that the potential of rOva to induce IL-2 secretion was retained and possibly even slightly enhanced after fusion to the TLR5-ligand rflaA. In contrast, LPS (data not shown) and rflaA alone, did not induce IL-2 secretion from Ova-specific T cells. Whereas the addition of LPS to rOva stimulated co-cultures decreased the rOva-induced IL-2 secretion (data not shown), a similar inhibitory effect was not observed for rflaA (Figure 33).
The patterns of IL-4 and IFN-γ secretion in the co-culture supernatants were very similar. The production of both cytokines was clearly attributed to $CD4^+$ T cells, since no cytokine

production could be detected in equally stimulated mDC cultures used as controls. For both cytokines rOva-induced secretion was inhibited by the addition of LPS (data not shown). At the concentration used (6.9 µg/ml) rflaA did not induce significant amounts of either IL-4 or IFN-γ. Furthermore, administrating the same amount of rflaA with rOva, no significant effects on rOva-induced cytokine secretion could be observed. Interestingly, stimulation with the rflaA:Ova fusion protein lead to a strong and highly significant reduction of the rOva induced production of Th1 and Th2 cytokines, IL-4 and IFN-γ, respectively. IL-4 secretion upon stimulation with rOva was reduced from 17 pg/ml to 0 to 3 pg/ml (for rflaA:Ova either non-modified or R/A), whereas IFN-γ secretion was reduced from approximately 2200 pg/ml for rOva to 300 to 600 pg/ml (for rflaA:Ova either non-modified or R/A) (Figure 33). Hereby, no significant differences between the non-modified and the reduced and alkylated fusion protein were observed (Figure 33). This reduction of IL-4 and IFN-γ correlated with the induction of IL-10 secretion by mDC upon stimulation with LPS (data not shown) and rflaA:Ova (200 to 220 pg/ml for either non-modified or R/A rflaA:Ova, Figure 33).

In accordance with the results obtained from stimulating mDC alone, the majority of IL-6 and IL-10 production in response to LPS (data not shown) and rflaA:Ova stimulation was assigned to mDC and not T cells. Furthermore, in the applied co-culture system the flagellin fusion constructs also revealed stronger mDC-derived IL-6 and IL-10 inducing capacities compared to flaA alone or an equimolar mixture of both components (Figure 33). Hereby, no significant differences were observed between IL-6 (ranging from 89 to 100 ng/ml) and IL-10 (200 to 220 pg/ml) secretion induced by rflaA:Ova either non-modified or R/A.

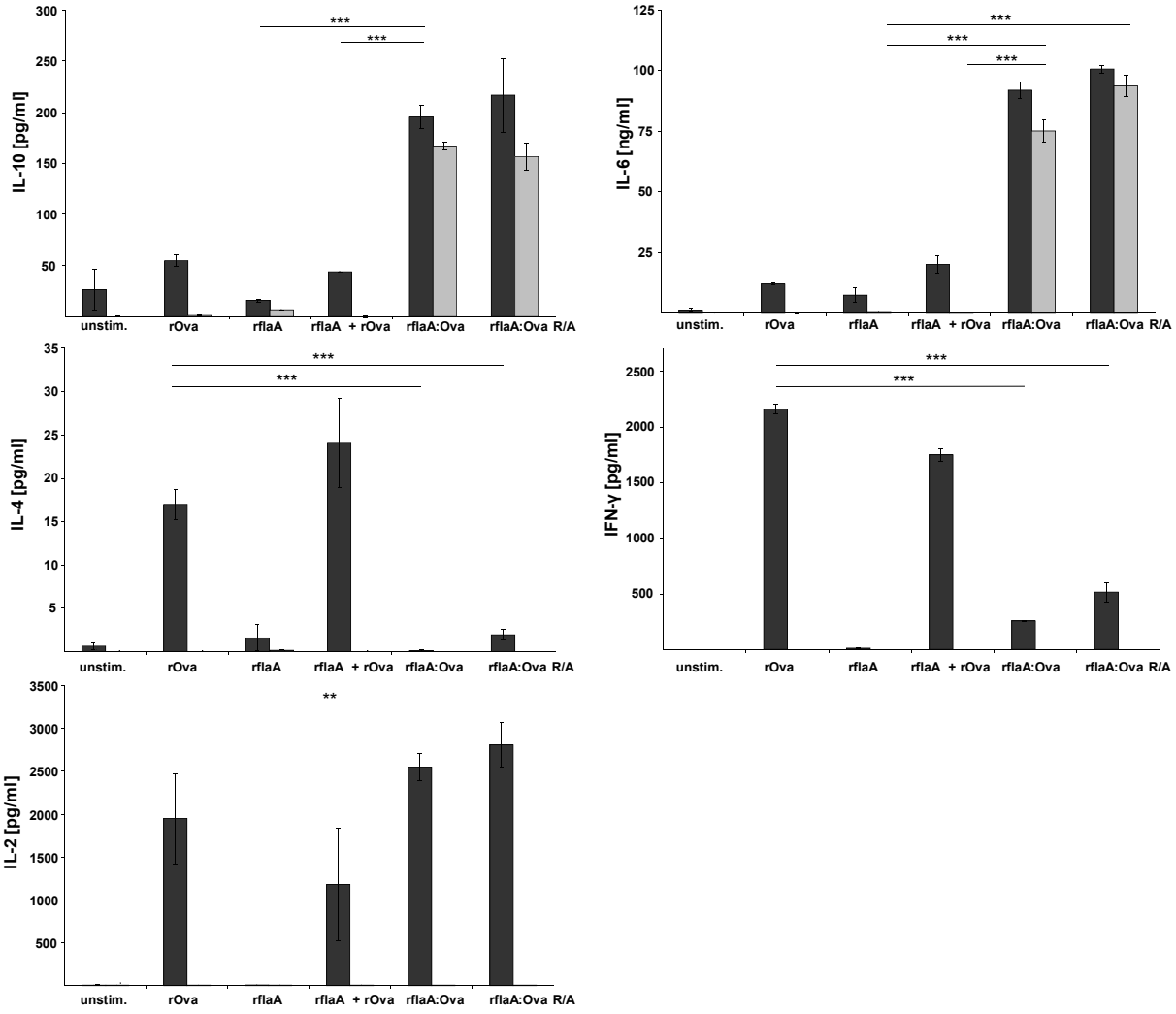


Figure 33: rflaA:Ova fusion proteins induce IL-2 production and repress IL-4 and IFN-γ production from naïve Ova-specific T cells. BALB/c mDC were cultured with (black bars) or without (grey bars) DO11.10 CD4 T cells, and stimulated with LPS (10 µg/ml, data not shown) and equimolar amounts of rOva (10 µg/ml), rflaA (6.9 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), rflaA:Ova (16.9 µg/ml), and rflaA:Ova (R/A) (16.9 µg/ml) for 72 h. IL-2 and IL-6 (after 24 h), IL-4, IL-10, and IFN-γ (after 72 h) were quantified by ELISA. Results are representative data taken from one out of three independent experiments ± SD.

4.2.10 rflaA:Ova does not induce Th17 development

The strong secretion of IL-6 observed upon stimulation with the fusion constructs (Figure 20 and Figure 33) suggested investigating whether the constructs might induce Th17 differentiation. Therefore, the secretion of TGF-β, and IL-23 from BALB/c mDC and IL-17A from DO11.10 CD4 T cells was investigated (Figure 34). Levels of IL-23 detected upon stimulation of BALB/c mDC with the different constructs were rather low. Hereby, both flagellin fusion proteins induced a slight IL-23 production of 30 to 80 pg/ml, comparable to the values observed upon administration of LPS (33 pg/ml, Figure 34 A). Moreover, comparison of TGF-β levels between mDC stimulated with the different constructs, revealed no difference

in TGF-β secretion (Figure 34 B). In co-culture experiments the slightly enhanced secretion of IL-23 upon stimulation with the rflaA:Ova fusion proteins did not result in significant differences between the rOva- (680 pg/ml) and rflaA:Ova (600 to 900 pg/ml, for either non-modified or R/A rflaA:Ova) induced IL-17A production (Figure 34 C).

Taken together, these results suggest that in the used *in vitro* co-culture system the administration of rflaA:Ova fusion proteins does not induce Th17-biased T cell responses.

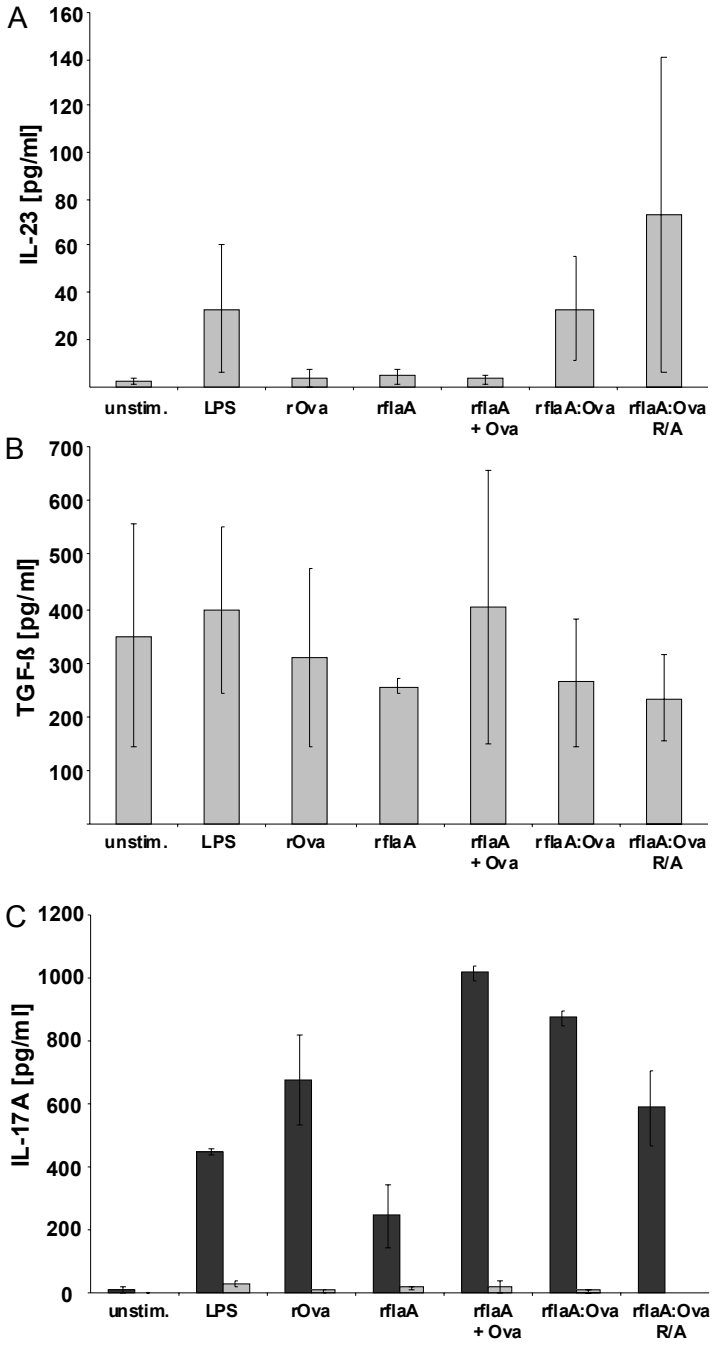


Figure 34: rflaA:Ova stimulation does not induce Th17-biased immune responses *in vitro*. BALB/c mDC were cultured with (black bars) or without (grey bars) DO11.10 CD4 T cells, stimulated with LPS (10 µg/ml) and equimolar amounts of rOva (10 µg/ml), rflaA (6.9 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), rflaA:Ova (16.9 µg/ml), and rflaA:Ova (R/A) (16.9 µg/ml). Supernatants were analyzed for IL-23 (A), TGF-β (B), and IL-17A (C) secretion. Results are mean (A and B) or representative (C) data from four independent experiments ± SD.

4.2.11 rflaA:Ova-induced IL-10 secretion modulates T cell-dependent cytokine production

To confirm whether rflaA:Ova-induced IL-10 secretion by mDC influenced T cell-dependent IL-4 and IFN-γ secretion in co-cultures, IL-10 production induced by rflaA:Ova stimulation was captured using a neutralizing antibody (Figure 35). In the *in vitro* system used, the addition of neutralizing antibody to IL-10 efficiently neutralized rflaA:Ova-induced IL-10 secretion, even when adding low amounts of neutralizing antibody (0.1 µg/ml, Figure 35 A). Furthermore, both IL-4 and IFN-γ secretion were strongly suppressed by the exogenous addition of rIL-10 (1 ng/ml). Upon addition of neutralizing αIL-10 to fusion protein stimulated co-cultures, levels of IFN-γ dose-dependently increased by up to 70% (from 579 to 990 pg/ml, Figure 35 B). Effects of IL-10 neutralizing antibody on IL-4 production were only modest, but a slight increase was observed when using the highest antibody concentration (Figure 35 C).

These results demonstrate that the rflaA:Ova-induced IL-10 secretion by mDC was affecting T cell-dependent cytokine production, suggesting the fusion proteins to be efficient immune modulators in the *in vitro* system applied.

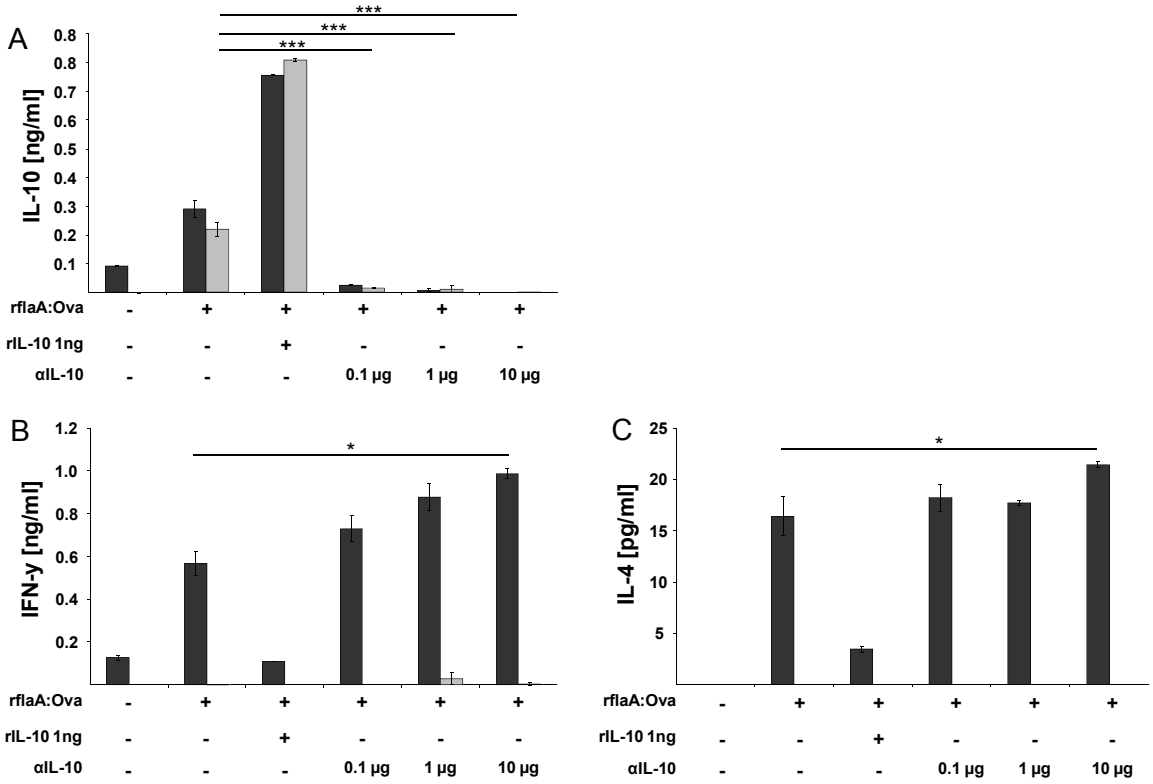


Figure 35: rflaA:Ova-induced IL-4 and IFN-γ production is diminished by mDC-derived IL-10. BALB/c mDC were cultured with (black bars) or without (grey bars) DO11.10 CD4 T cells and stimulated with rflaA:Ova (16.9 µg/ml) for 72 h in the presence of rIL-10 or neutralizing αIL-10, respectively. Culture supernatants were analyzed for IL-10 (A), IFN-γ (B), and IL-4 (C) secretion. Results are representative data taken from one out of three independent experiments ± SD.

4.2.12 rflaA:Ova mediated immune modulation is mouse strain independent

To further investigate the mechanism behind the observed immune modulating effects of the rflaA:Ova fusion proteins by using knock out mice (C57BL/6 background), experiments were repeated with immune cells derived from C57BL/6 mice (Figure 36 to Figure 38). Since T cells from C57BL/6 mice strain are strongly Th1-biased (179), Ova-specific OT-II CD4 T cells did not produce significant amounts of Th2 cytokines IL-4 and IL-5 upon stimulation with rOva or rflaA:Ova (data not shown), whereas levels of IL-6, IL-10, and IFN-y were comparable to the values observed in BALB/c mice (Figure 20, Figure 33). Therefore, further experiments focused on the potential suppression of Th1-responses by investigating IFN-y secretion and IL-10 production by mDC (Figure 36 and Figure 37). Additionally, since both the non-modified and the reduced and alkylated fusion protein displayed the same immunologic properties tests were restricted to the non-modifed rflaA:Ova.

Stimulation of C57BL/6-derived mDC with the different constructs revealed that, rflaA:Ova induced a dose-dependent IL-6 and IL-10 secretion from C57BL/6 mDC (Figure 36 A and B). Hereby, levels of IL-6 (85 ng/ml) and IL-10 (400 pg/ml) secretion upon stimulation with the rflaA:Ova concentration primarily used in previous experiments (16.9 µg/ml) were comparable to the results obtained from BALB/c mice (50 to 75 ng/ml IL-6, approx 200 pg/ml IL-10, see Figure 20 and Figure 36). Again, stimulation with the equimolar mixture of both proteins did not induce comparable cytokine secretion.

In co-culture experiments of C57BL/6 mDC with Ova-specific OT-II CD4 T cells stimulation with the specific T cell antigen rOva dose dependently induced IFN-γ secretion from OT-II T cells (Figure 37 A). This Ova-dependent IFN-γ secretion was neither enhanced nor repressed by co-administration of rflaA. Interestingly, increasing concentrations of rflaA:Ova dose-dependently repressed IFN-γ production (to 0.5 ng/ml), whereas lower doses of rflaA:Ova did not (2.6 ng/ml IFN-γ upon stimulation with 1 mg rflaA:Ova, Figure 37 A). Using cells from a C57BL/6 background, the absolute amounts of IL-2 (<20 pg/ml) secreted by Ova-specific T cells were rather low. However, upon administration of rflaA:Ova higher levels of IL-2 secretion (10 pg/ml for 5 µg/ml rflaA:Ova) were observed compared to equimolar amounts of rOva alone (3 pg/ml) or rflaA and rOva applied as a mixture (3 pg/ml, Figure 37 B). In line with the results obtained for IFN-γ, co-administration of rflaA and rOva did not afffect the rOva-induced IL-2 secretion.

In accordance with the results obtained using cells from a BALB/c background (see 4.2.12), the repression of IFN-γ upon stimulation with rflaA:Ova (Figure 37 A) was associated with a considerable induction of IL-10 secretion (up to 500 pg/ml IL-10), whereas rOva alone, or mixed with rflaA did not induce IL-10 secretion (Figure 37 C).

In summary, the dose-dependent suppression of Ova-induced IFN-γ secretion in C57BL/6 derived T cells, likely is mediated by rflaA:Ova-induced IL-10 secretion from mDC. The results are in accordance with the results obtained for BALB/c mice, suggesting the same mode of action in both mouse strains.

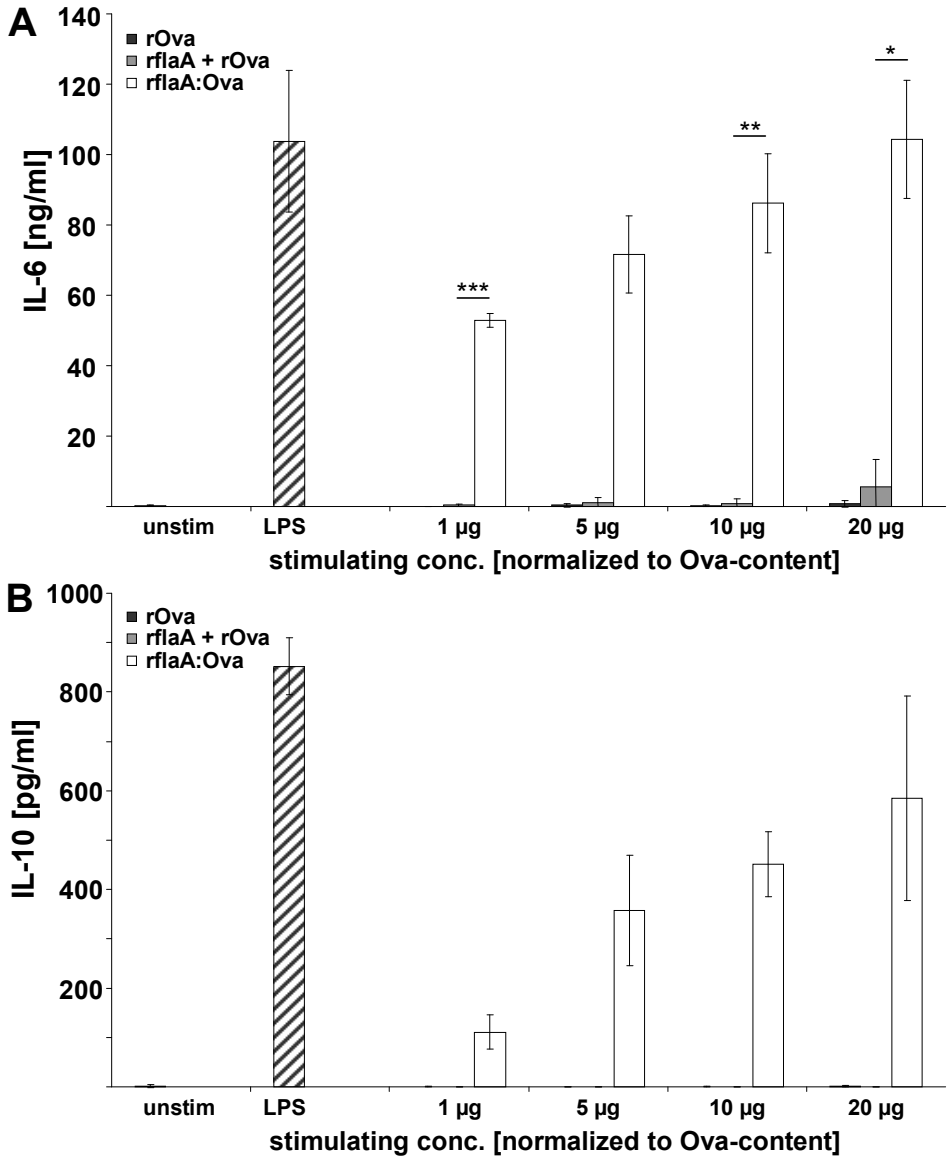


Figure 36: rflaA:Ova induces IL-6 and IL-10 secretion from C57BL/6 mDC. C57BL/6 mDC were stimulated with LPS (10 µg/ml) and the indicated equimolar amounts of rOva (black), rflaA plus rOva (grey), or rflaA:Ova (white) for 72 h. Levels of IL-6 (A) and IL-10 (B) were determined by ELISA. Data are mean of three independent experiments ± SD.

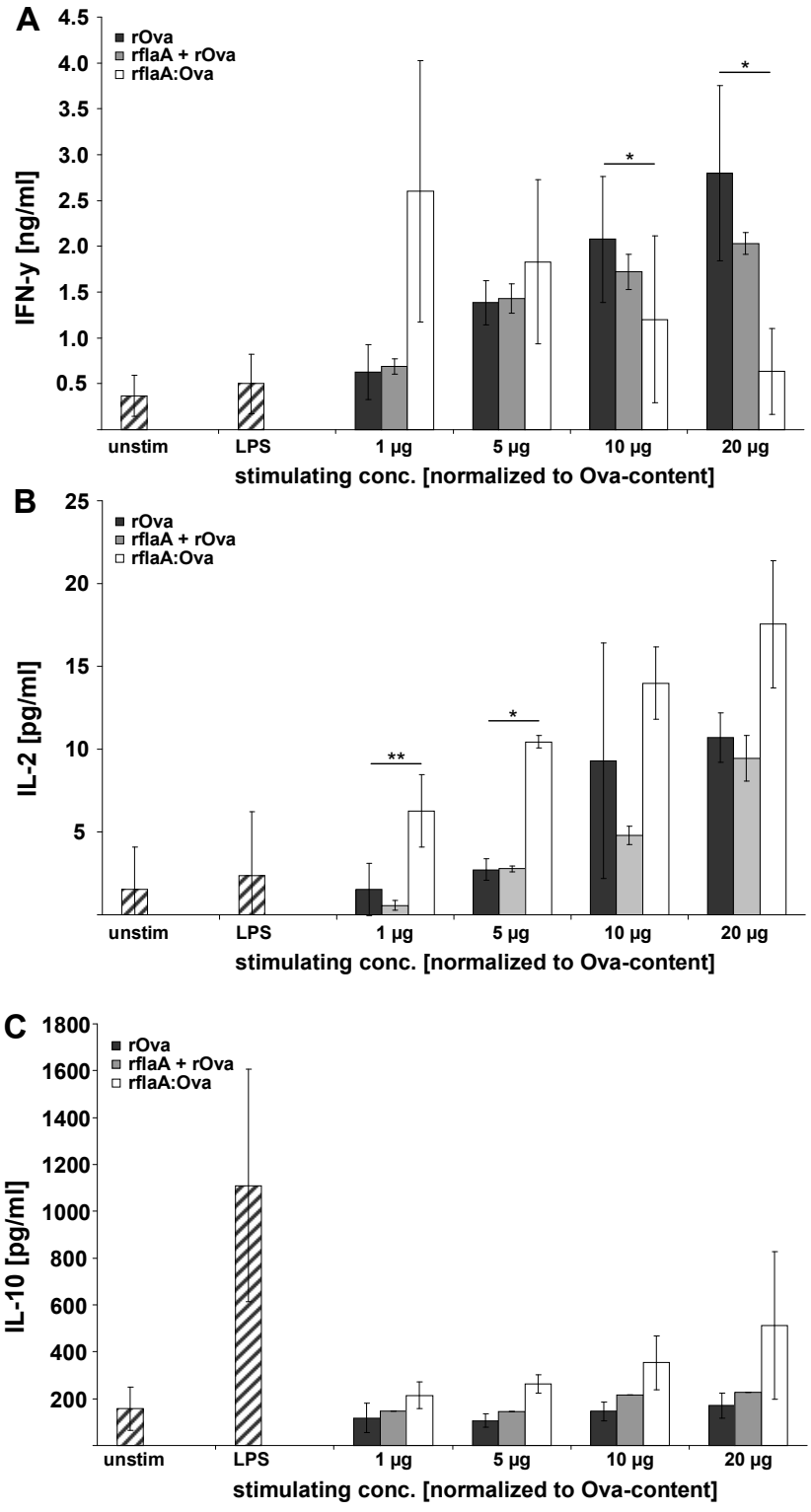


Figure 37: rflaA:Ova modulates OT-II T cell differentiation. C57BL/6 mDC were co-cultured with OT-II CD4 T cells and stimulated with LPS (10 µg/ml) and the indicated equimolar amounts of rOva (black), rflaA plus rOva (grey), or rflaA:Ova (white) for 72 h. Levels of IFN-γ (A), IL-2 (B), and IL-10 (C) were determined by ELISA. Data are mean of three independent experiments ± SD.

In line with this and the results presented for BALB/c derived mDC (Figure 35), rflaA:Ova-induced IL-10 secretion by C57BL/6 mDC was neutralized by co-administration of neutralizing αIL-10 antibody (Figure 38 A). In accordance with the results obtained using BALB/c derived mDC and DO11.10 CD4 T cells the inhibition of secreted IL-10 resulted in a 2.5-fold enhanced IFN-γ production by the Ova-specific OT-II T cells (Figure 38 B), whereas rflaA:Ova mediated IL-6 secretion was not affected (Figure 38 C).

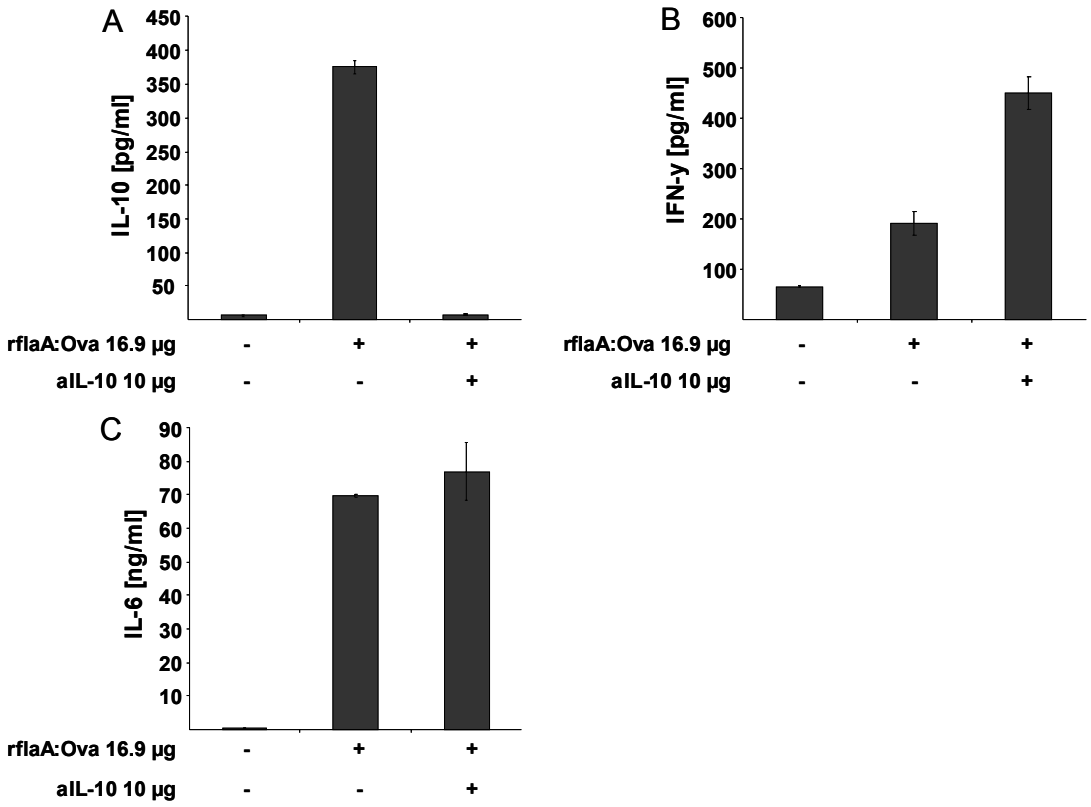


Figure 38: Neutralization of rflaA:Ova-induced IL-10 secretion restores IFN-γ secretion but does not affect IL-6 secretion. C57BL/6 mDC were co-cultured with OT-II CD4 T cells and stimulated with rflaA:Ova in the presence or absence of neutralizing αIL-10 for 72 h. Levels of IL-10 (A), IFN-γ (B), and IL-6 (C) were determined by ELISA. Results are mean of two independent experiments ± SD.

4.2.13 flaA:Ova-induced cytokine secretion depends on TLR-signalling

In order to confirm the contribution of TLR-signalling to the observed effects, the rflaA:Ova-induced cytokine secretion was compared between mDC derived from C57BL/6 and mice deficient for the adaptor protein of TLR signaling MyD88 ($MyD88^{-/-}$, Figure 39). Kock out of MyD88 mediated signalling was sufficient to abrogate mDC derived production of IL-6 and IL-10 (Figure 39 A and B). In accordance with the results presented above for C57BL/6 derived cells (Figure 36) neither rflaA nor rOva provided alone or as an equimolar mixture did induce significant IL-6 or IL-10 secretion from $MyD88^{-/-}$ mDC.

In co-cultures of Ova-specific OT-II CD4 T cells with mDC deficient for MyD88 no difference in IFN-γ secretion was observed upon stimulation with either rOva, or rflaA mixed with rOva, both inducing approximately 2.25 to 2.45 ng/ml IFN-γ (Figure 39 C). Interestingly, using MyD88 deficient mDC the observed repression of IFN-γ secretion upon stimulation with the fusion construct (using wild type cells, approximately 1.2 ng/ml) was rescued leading to a secretion of 2.5 ng/ml IFN-y which was also observed for either rOva alone or the equimolar mixture of both components. Taken together, these results suggest the immune modulating properties of the fusion protein to be dependent on TLR-mediated signaling.

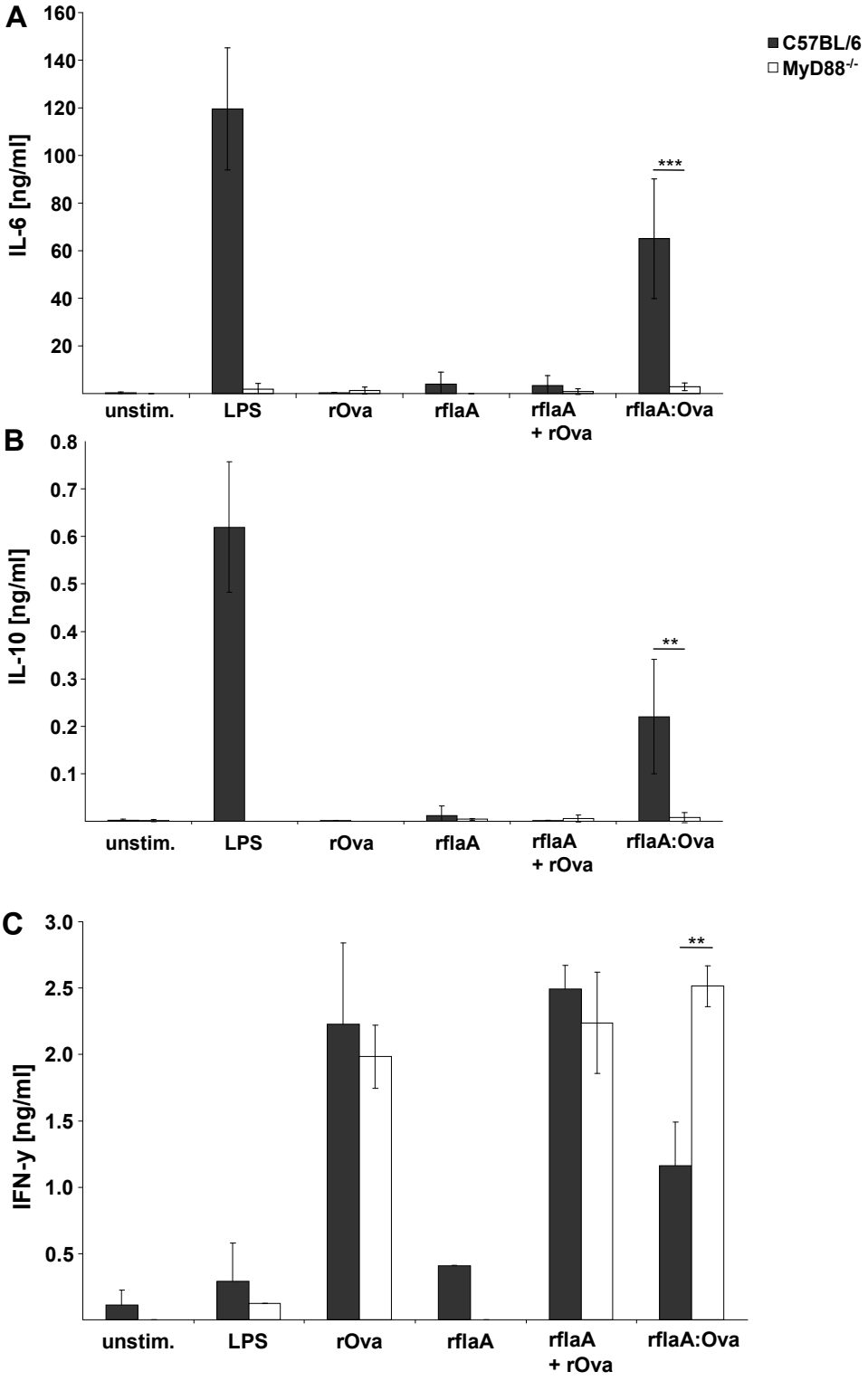


Figure 39: The immune modulating properties of rflaA:Ova are dependent on TLR-signalling. C57BL/6 (black) or MyD88$^{-/-}$ (white) mDC were stimulated with LPS (10 µg/ml) or equimolar amounts of rOva (10 µg/ml), rflaA (6.9 µg/ml), rflaA (6.9 µg/ml) plus rOva (10 µg/ml), and rflaA:Ova (16.9 µg/ml). Culture supernatants were collected after 72 h, and concentrations of IL-6 (A) and IL-10 (B) were determined by ELISA. For measurement of IFN-γ (C) mDC were co-cultured with OT-II CD4 T cells. Data are mean of two independent experiments ± SD.

4.2.14 rflaA:Ova is also able to suppress Th1 and Th2 cytokine secretion from Th2-biased T cells

After showing the fusion protein to be able to suppress cytokine secretion from naïve antigen-specific T cells, the immune modulatory effect of rflaA:Ova on *in vivo* Th2-primed T cells was examined. Therefore, CD4 T cells were isolated from spleens of BALB/c mice immunized with Ova and alum (see 3.3.1, Figure 40). When co-culturing Th2-primed CD4 T cells with BALB/c mDC, Ova restimulation dose-dependently induced secretion of Th2- (IL-4: up to 11 pg/ml and IL-5: up to 139 pg/ml) and Th1-cytokines (IFN-γ: up to 248 pg/ml), whereas stimulation with rflaA:Ova only induced lower cytokine secretion (IL-4: 0.89 pg/ml, IL-5: 38 pg/ml, IFN-γ:11.9 pg/ml, Figure 40). Interestingly, when Ova and rflaA:Ova were provided simultaneously to co-cultures containing Ova-primed CD4 T cells a significant (mixed linear model with mixed factor treatment group and a random factor assay, see 3.4) reduction of Ova-induced Th2 and Th2 cytokine secretion was observed compared to rOva-stimulation alone. For the highest concentration of Ova used (200 µg/ml) cytokine secretion was reduced by a factor of 1.83 for IL-4, 2.39 for IL-5, and 3.54 for IFN-γ compared to stimulation with Ova alone (Figure 40). This is especially interesting since these reductions were observed although Ova was used in a 20-fold molar excess to rflaA:Ova (200 µg Ova vs. 16.9 µg rflaA:Ova).

Therefore, it was clearly shown that the rflaA:Ova fusion protein was capable of repressing cytokine secretion not only from naïve but also from *in vivo* Th2-primed T cells.

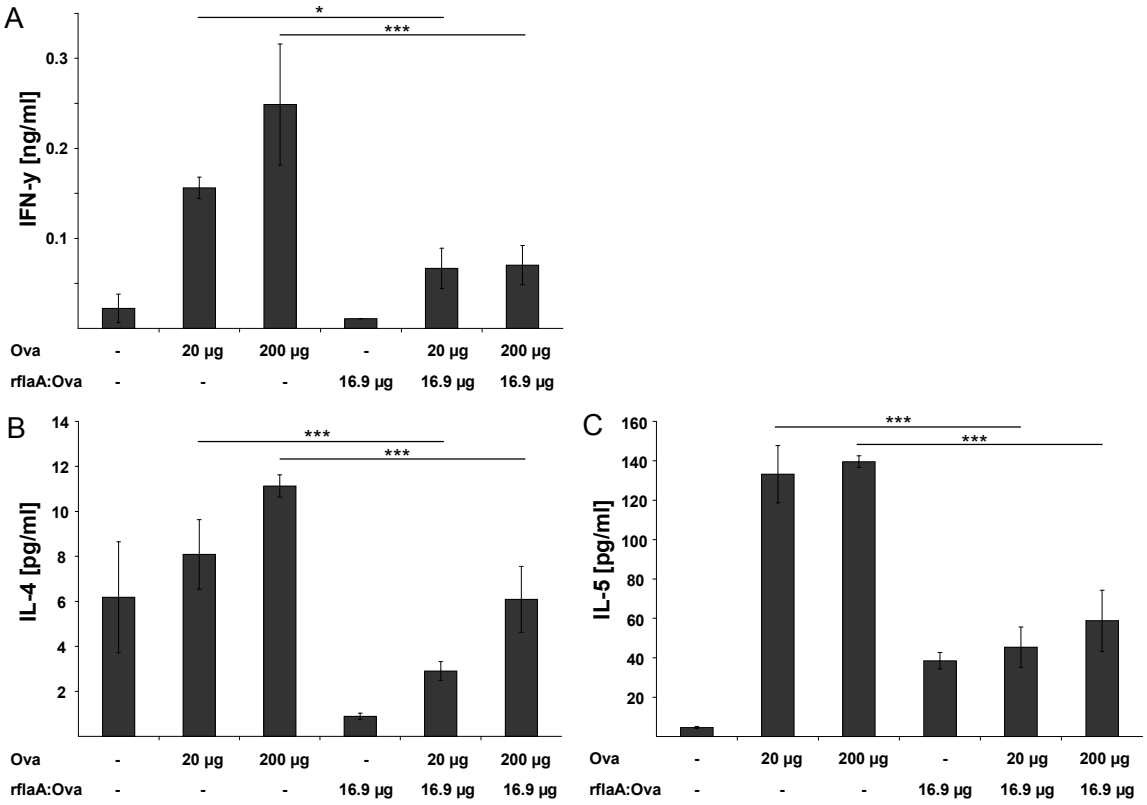


Figure 40: rflaA:Ova suppresses cytokine secretion from *in vivo* primed Th2-biased T cells. CD4 T cells from Ova-immunized BALB/c mice were co-cultured with BALB/c mDC, and stimulated with rOva, and/or rflaA:Ova for 72 h. Th2 cytokines IL-4 and IL-5, and Th1 cytokine IFN-y in the culture supernatant were quantified by ELISA. Results are mean of two independent experiments ± SD.

4.2.15 Blocking of endocytosis prevents rflaA:Ova-induced cytokine secretion

In order to investigate whether increased uptake of the fusion protein might initially account for the observed stronger effects of the fusion protein compared to rflaA alone or the mixture of both components, mDCs were stimulated with rflaA:Ova in the presence of inhibitors of actin-polymerization (cytochalasin D) and lysosomal fusion (bafilomycin A1) (see 3.2.4, Figure 41).

Blocking of both actin-polymerization by cytochalasin D and lysosomal fusion by bafilomycin A1 decerased rflaA:Ova-induced IL-6 secretion from mDC by 40% (CytD) and 55% (BafA1), respectively. Noteworthy, simultaneous co-administration of both inhibitors enhanced the effect to 70% suppression of rflaA:Ova-induced IL-6 secretion (Figure 41). DMSO used as solvent for the inhibitors had no impact on fusion protein induced IL-6 secretion (Figure 41). Since stimulation with equimolar amounts of rflaA alone did not induce IL-6 secretion the influence of cytochalasin D and bafilomycin A1 pretreatment on rflaA mediated mDC cytokine secretion could not be tested in this system.

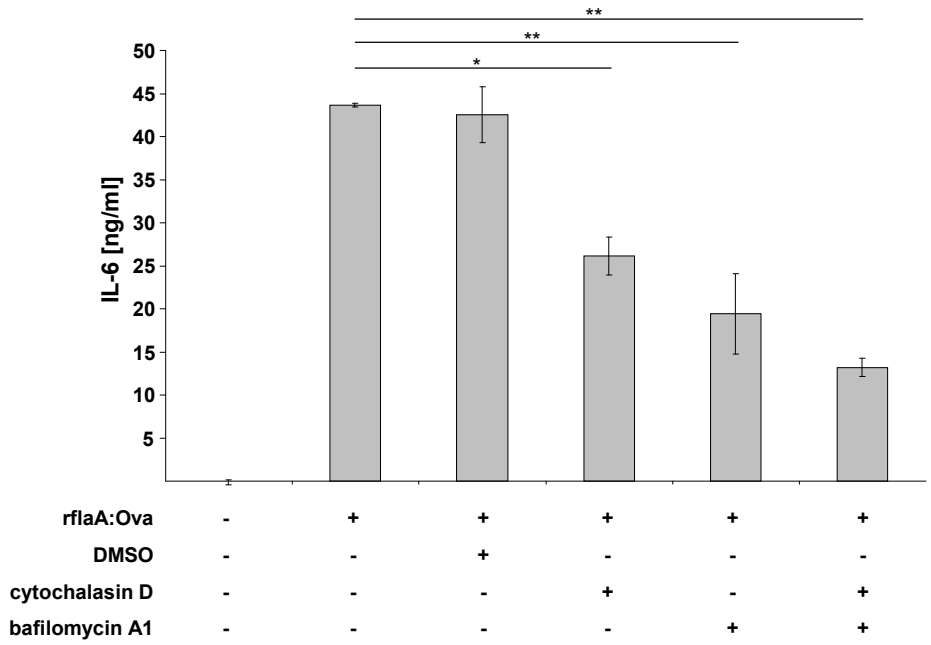


Figure 41: rflaA:Ova-induced IL-6 secretion is dependent on endocytosis. BALB/c mDC were preincubated with cytochalasin D (5 µg/ml) and/or bafilomycin A1 (100 ng/ml) for one hour. Subsequently, cells were stimulated rflaA:Ova (16.9 µg/ml). The levels of IL-6 in the culture supernatant after 24 h were determined by ELISA. Results are mean values ± SD of two independent experiments.

Experiments were repeated using C57BL/6 derived mDC (Figure 42). Whereas in this mouse strain the inhibitors bafilomycin A1 and cytochalasin D had no effect on rflaA:Ova-induced IL-6 secretion, pretreatment with chloroquine dose-dependently repressed IL-6 secretion (Figure 42). The effects of chloroquine on BALB/c mDC were not tested.

In summary, these results suggest that endocytotic uptake of the fusion protein might be due to a different mechanism but may contribute at least in part to the observed stronger mDC activation.

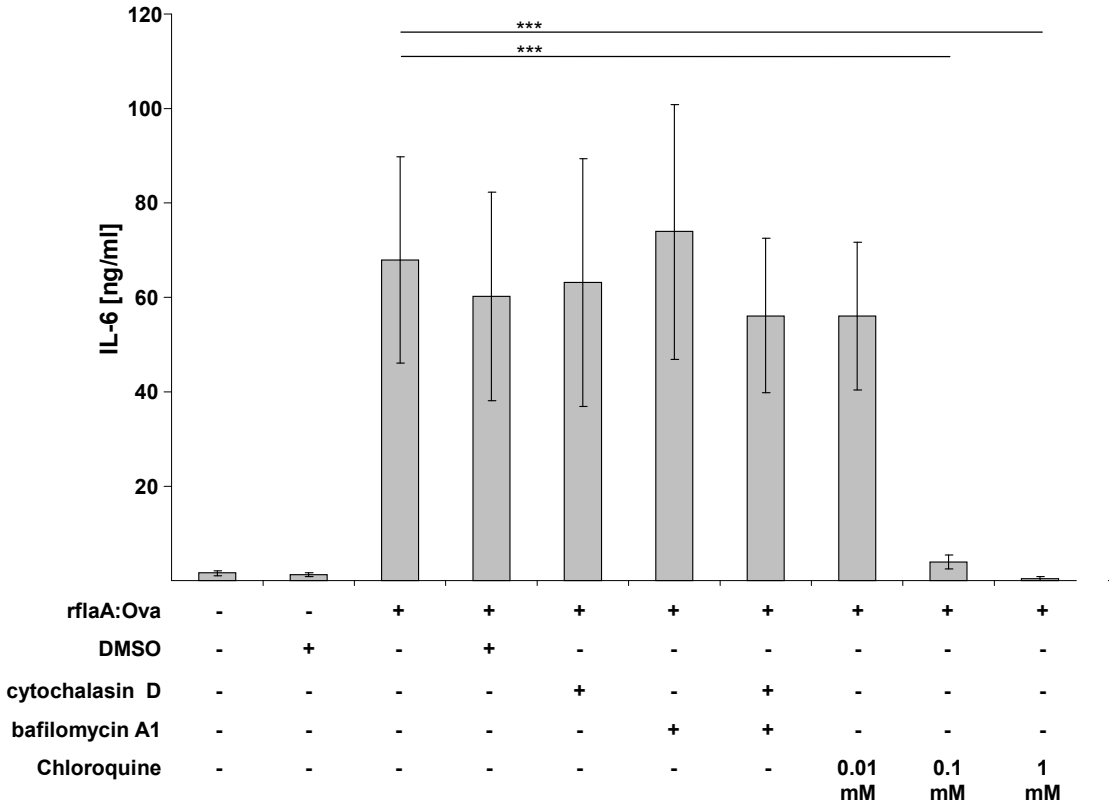


Figure 42: Chloroquine suppresses rflaA:Ova-induced cytokine secretion from C57BL/6 mDC. C57BL/6 mDC were pretreated with inhibitors cytochalasin D (5.0 µg/ml), bafilomycin A1 (100 ng/ml) and/or chloroquine (at the indicated concentrations) for 1 h and then stimulated with rflaA:Ova (4.88 µg) for 24 h. Supernatants were collected and checked for the levels of IL-6 by ELISA. Data are mean of four independent experiments ± SD.

4.2.16 Activation of mDC by flagellin:allergen fusion proteins

In order to prove whether the results obtained for rflaA:Ova can be transferred to other allergens, fusion proteins consisting of flagellin and the major peanut (Ara h 2) and peach (Pru p 3) allergens, rflaA:Ara h 2 and rflaA:Pru p 3 were generated and compared for their ability to activate C57BL/6 mDC and induce cytokine secretion (Figure 43 and Figure 44). In accordance with the results obtained for rflaA:Ova, IL-1β and IL-6 production by mDC was 4.5-fold higher when stimulated with rflaA:Pru p 3 than for the equimolar amounts of rflaA alone or mixed with Pru p 3 (250 pg/ml IL-1ß for 26 µg rflaA:Pru p 3 vs 55 pg/ml for the equimolar mixture, Figure 43 A). Suprisingly, the rflaA:Ara h 2 fusion protein in general proved to be a rather weak inducer of cytokine secretion, however using 32 µg of rflaA:Ara h 2 a slight production of IL-6 (10 ng/ml) and IL-1β (45 pg/ml) was observed (Figure 43). IL-10 secretion from C57BL/6 mDC was only detected upon stimulation with LPS and to a lower extend rflaA:Pru p 3 (approximately 100 pg/ml, Figure 43 C). Flagellin stimulation induced dose-dependent IL-1β and IL-6, but no IL-10 secretion, which was weaker than for the investigated fusion constructs. In accordance with the results presented above, IL-23 levels upon stimulation with the different constructs were very low near the limit of detection of the used ELISA. Among the tested constructs only rflaA:Ova induced minimal amounts of IL-23 (approximately 17 pg/ml) (data not shown). Furthermore, none of the used purified allergens (rPru p 3 or rAra h 2) did induce cytokine secretion from C57BL/6 mDC (Figure 43).

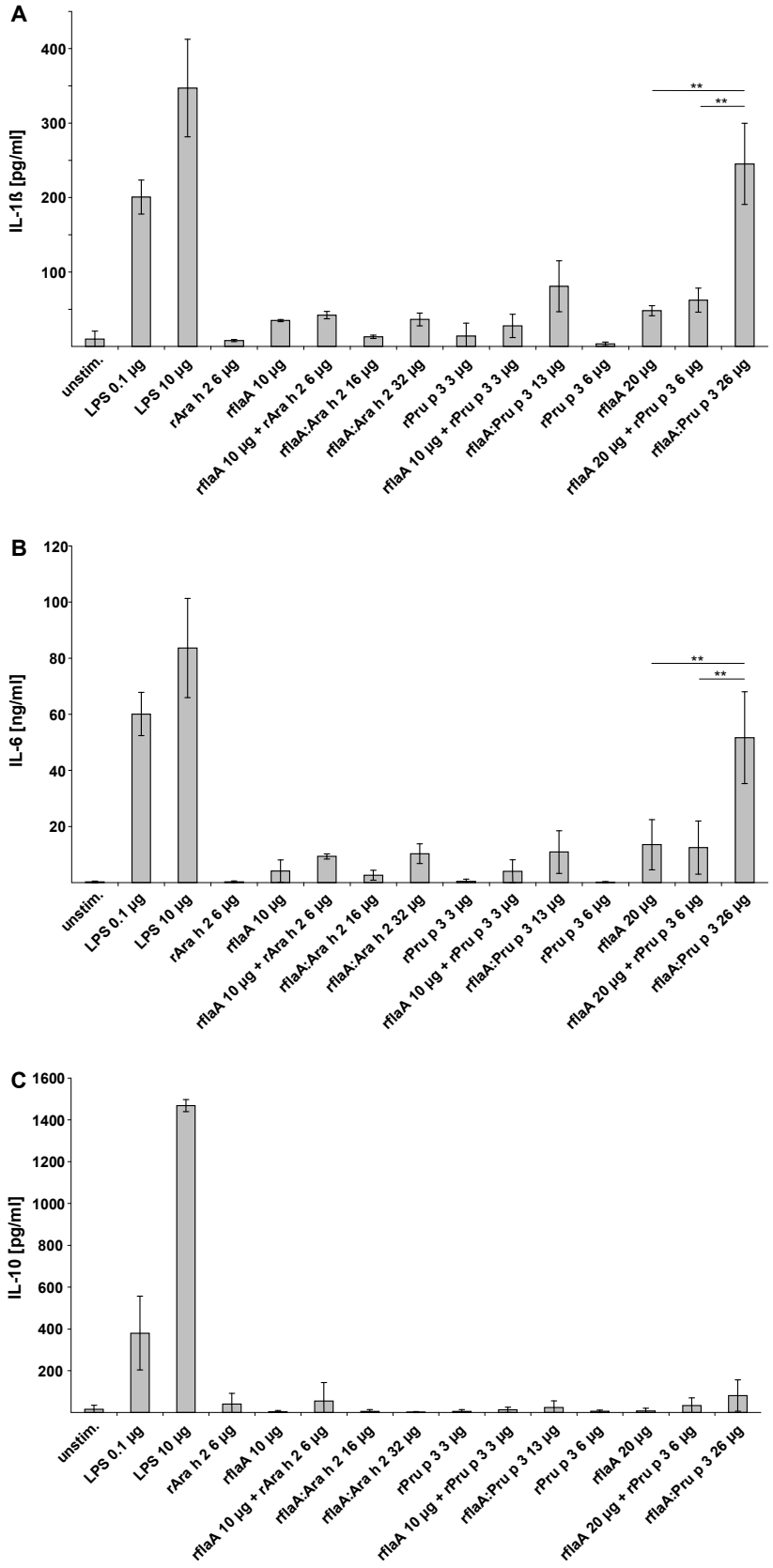


Figure 43: Cytokine secretion from mDC induced by the different fusion proteins. C57BL/6 mDC were stimulated with the indicated constructs for 24 h. Supernatants were analyzed for levels of IL-1β (A), IL-6 (B), and IL-10 (C). Data are mean ± SD of three independent experiments.

All three fusion constructs induced strong mDC activation as determined by CD40 upregulation (Figure 44). This mDC activation was still observed in Trif$^{-/-}$ cells but not in MyD88$^{-/-}$Trif$^{-/-}$-derived cells (Figure 45). In line with the ELISA results presented above (Figure 43) LPS and rflaA did induce mDC activation whereas stimulation with the different allergens alone did not activate mDC (Figure 44). When testing for TLR5 upregulation upon stimulation with the different fusion proteins or rflaA, only rflaA:Ova-induced TLR5 upregulation on the surface of C57BL/6 mDC, which was abrogated in Trif$^{-/-}$ and MyD88$^{-/-}$Trif$^{-/-}$ deficient cells (Figure 45).

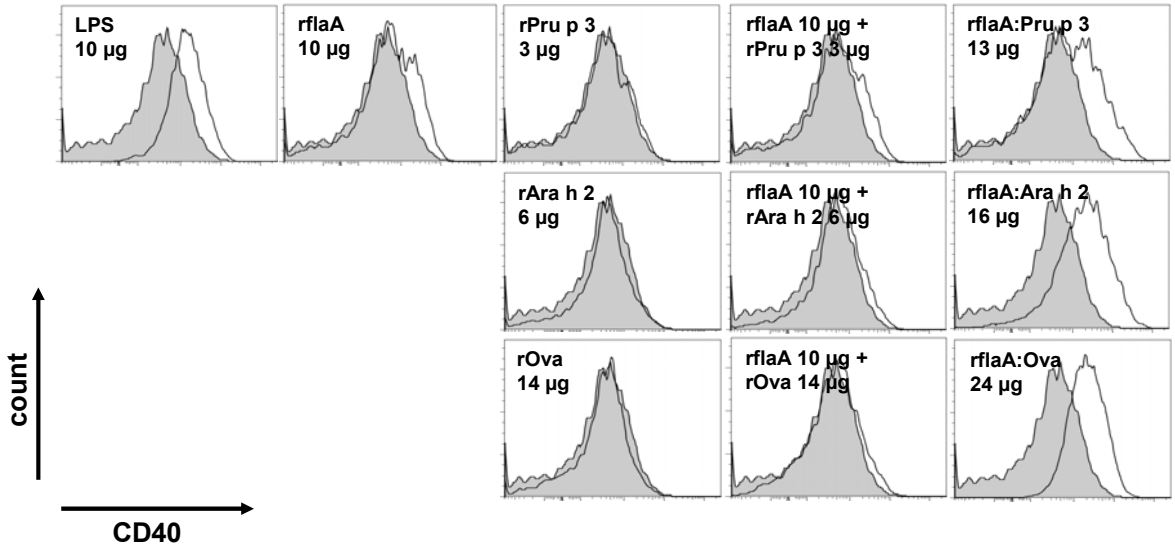


Figure 44: Flagellin:allergen fusion proteins activate C57BL/6 mDC. C57BL/6 mDC were stimulated with the indicated constructs for 24 h. Cells were harvested, washed, and stained for CD11b, CD11c, B220, CD40; cells were gated on CD11b^{+}CD11c^{+}B220^{-} mDC and analyzed for CD40 expression. Grey solid lines: unstimulated, black lines: stimulated with indicated construct. Data are representative results taken from one out of two independent experiments.

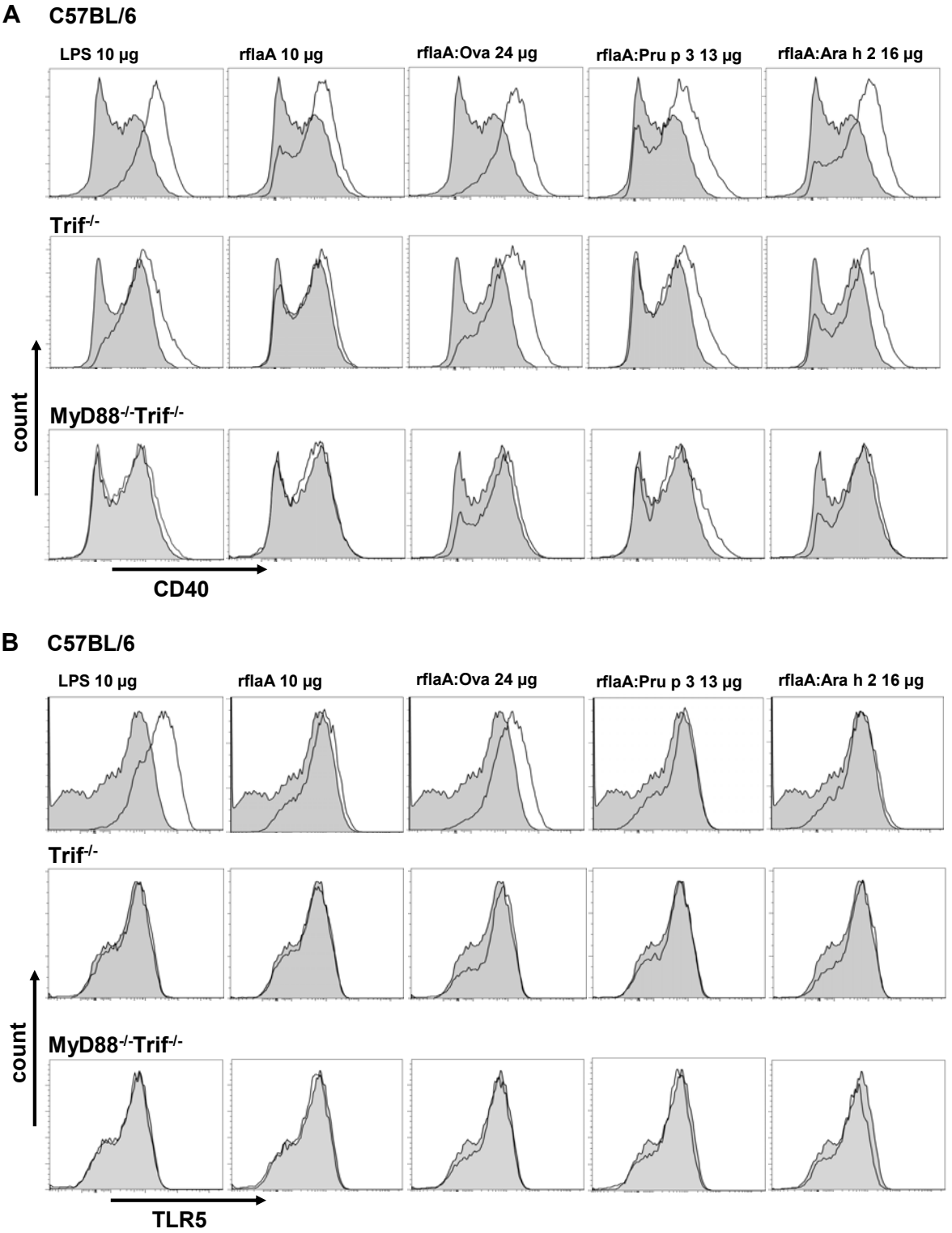


Figure 45: Flagellin A and flagellin:allergen fusion proteins mediated activation of C57BL/6 mDC depends on TLR signalling. C57BL/6 mDC were stimulated with the indicated constructs for 24 h. Cells were harvested, washed, and stained for CD11b, CD11c, B220, CD40 (A), and TLR5 (B); cells were gated on $CD11b^{+}CD11c^{+}B220^{-}$ mDC and analyzed for CD40 (A) and TLR (B) expression. Grey solid lines: unstimulated, black: stimulated with indicated construct. Data are representative results taken from one out of two independent experiments.

4.3 rflaA:Ova prevents intestinal allergy in mice

4.3.1 rflaA and rflaA:Ova activate DC and induce cytokine secretion in vivo

In order to investigate the effect of the rflaA:Ova fusion protein *in vivo* equimolar amounts of rflaA, rOva, rflaA plus Ova, and the fusion construct rflaA:Ova were injected i.p. into BALB/c mice. 24 h post injection splenic DC activation and serum cytokine levels were investigated (Figure 46, data not shown). Upon i.p.-administration of equimolar amounts of rflaA, rflaA plus Ova, and the fusion protein rflaA:Ova splenic DC activation was observed to a similar extend, whereas rOva alone did not activate splenic DCs (Figure 46). For the different constructs two concentrations were tested, either equimolar to 10 or 20 µg Ova. Hereby, higher protein amounts resulted in better splenic DC activation and were depicted in Figure 46. The activation markers CD40, CD69 and CD80 showed the same pattern (Figure 46 A, CD80 and CD40 data not shown), whereas for CD86 a stronger DC activation was observed upon rflaA administration. Noteworthy, due to unknown reasons both rflaA plus Ova and rflaA:Ova injection induced only DC activation in two out of three mice (Figure 46).

In a second experiment serum samples of BALB/c mice treated by i.p.-injection were investigated for cytokine secretion by multiplex ELISA. However, pronounced devations were observed between individual animals within the different treatment groups which did not allow detecting differences between the groups. Noteworthy, rflaA:Ova treatment induced a slightly elevated GM-CSF production in serum samples of four out of four animals, whereas for the other groups only one or two out of four mice showed GM-CSF induction (data not shown. Moreover, no differences in serum cytokine levels between the different groups were observed for IL-2, IL-5, IL-13, IL-18, and MCP-1 (data not shown). Furthermore IL-1α, IL-4, IL-6, IL-10, IL-17, IL-23, IFN-γ, and TNF-α were not detectable (data not shown).

To further investigate the effects of rflaA:Ova administration BALB/c mice were injected with different amounts od rflaA:Ova intraperitoneal, sera were collected 24 h post injection, and checked for cytokine production (Figure 47). Interestingly, among the tested concentrations of rflaA:Ova the lowest concentration (0.17 µg) induced the strongest release of IL-1α, IL-10, IL-18, IL-22, IFN-γ (Figure 47), and MCP-1 (data not shown). In accordance with the results presented above, high heterogeneities were observed between individual animals within one group (Figure 47). Median IL-2 and IL-5 levels were elevated in rflaA:Ova-treated animals. Hereby no differences between the used concentrations were detected. Furthermore, nearly no production of IL-6 was observed 24 h post injection. However, again pronounced heterogeneities among single mice were observed (Figure 47). No difference compared to

PBS-treated animals was observed for IL-13 and no cytokine production was detectable for the cytokines IL-4, IL-17, IL-23, and TNF-α (data not shown).

In summary, due to the pronounced heterogeneities, the results obtained by multiplex ELISA delivered no evidence for a strong immune activation by the different constructs at the investigated time point 24 h post i.p.-injection.

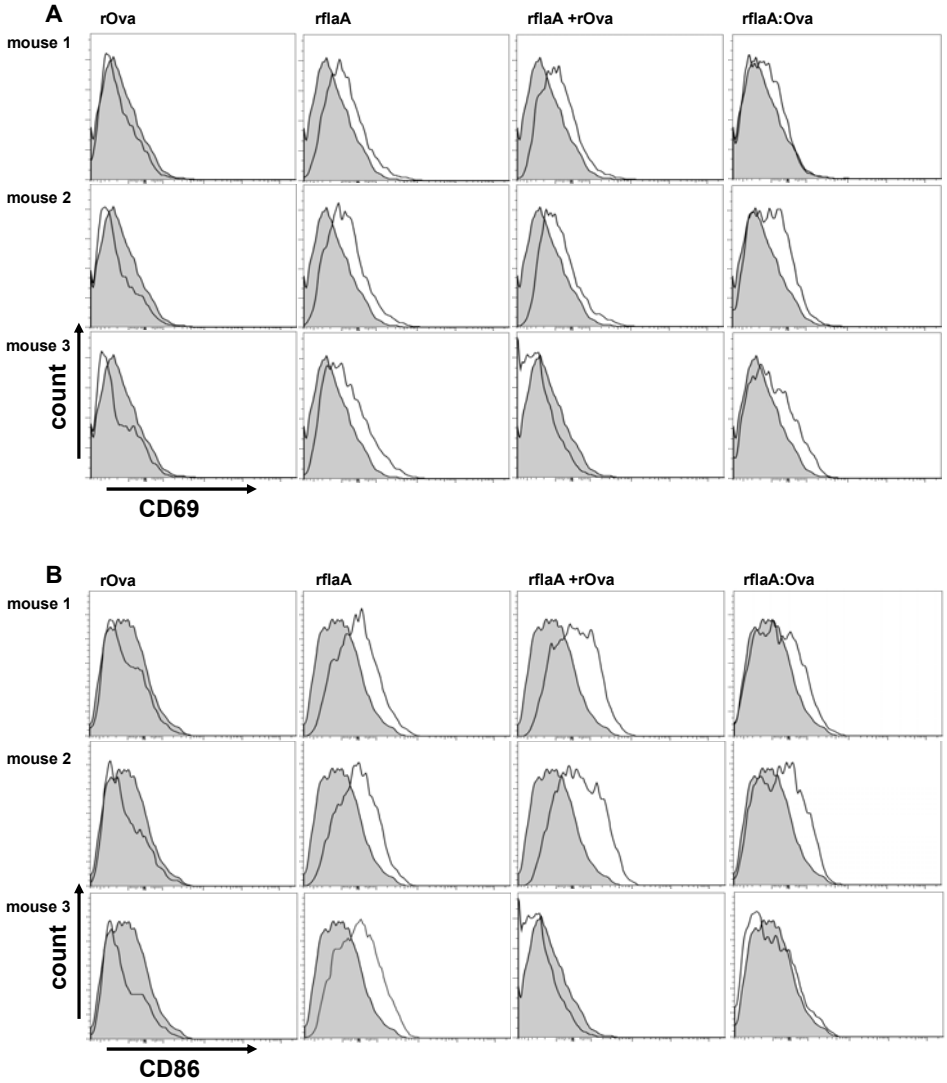


Figure 46: rflaA and rflaA:Ova induce splenic DC activation. BALB/c mice (n = 3 per group) were immunized with PBS, rflaA (13.8 µg), rOva (20 µg), rflaA (13.8 µg) + rOva (20 µg), and rflaA:Ova (33.8 µg) by i.p.-injection. 24 h post injection spleens were removed and digested with collagenase D. Spleen cells were stained for CD11b, CD11c, B220, CD69 (A) and CD86 (B) and DC activation was investigated by flow cytometry. Cells were gated on $CD11c^{+}B220^{-}$ fraction. Grey shaded: representative PBS control (taken from one out of three mice), black lines: stimulated with indicated construct.

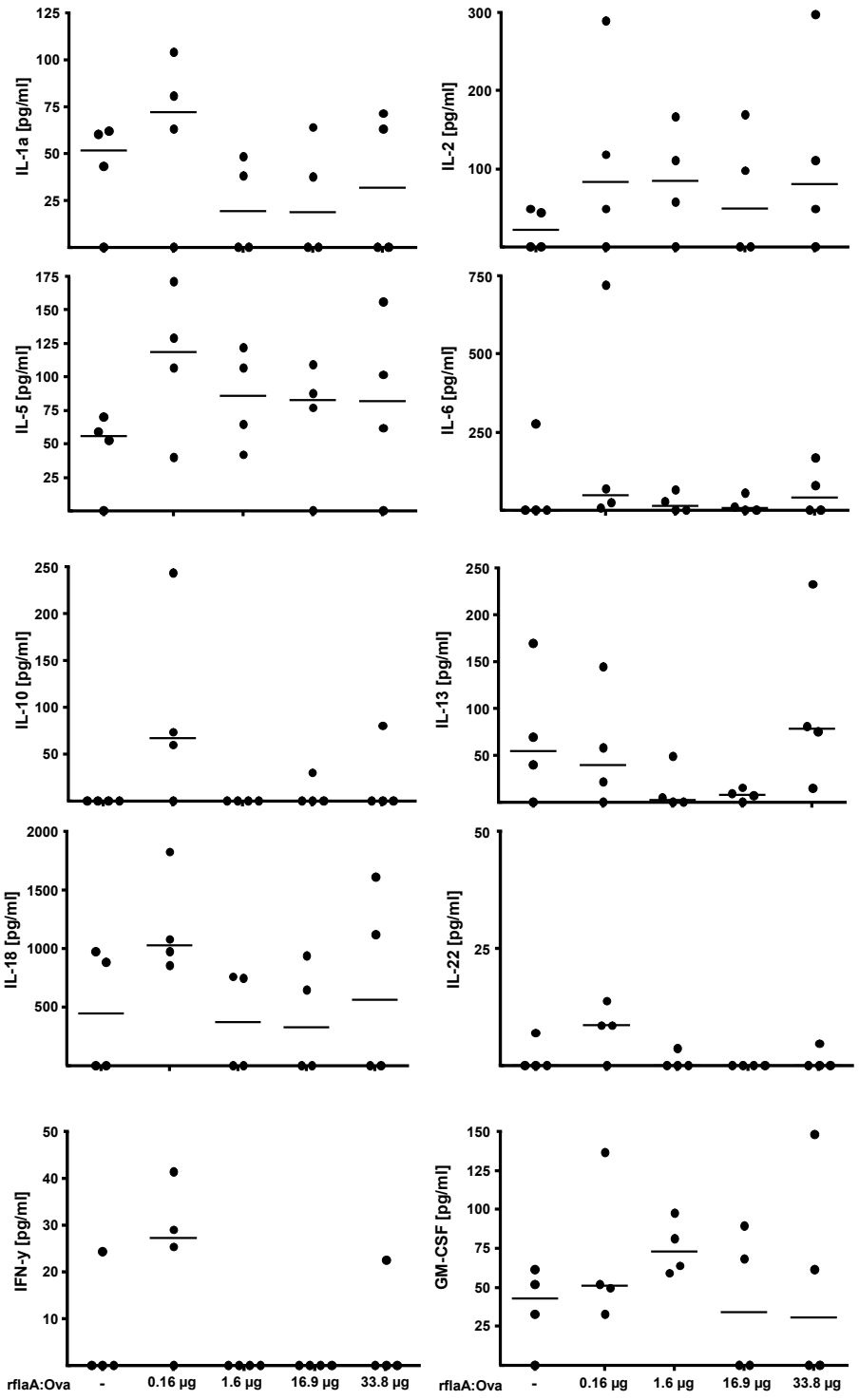


Figure 47: Cytokine secretion induced by rflaA:Ova *in vivo*. BALB/c mice (n = 4 per group) were treated with the indicated amounts of rflaA:Ova by i.p.-injection in sterile PBS. 24 h post injection serum samples were obtained by cardiac puncture in deep anaesthesia. Median Cytokine levels in sera were determined by multiplex ELISA.

4.3.2 Evaluation of rflaA and rflaA:Ova as prophylactic vaccines in the in vivo model of Ova-induced intestinal allergy

In a first vaccination study the potential of rflaA and rflaA:Ova were evaluated in a prophylactic vaccination approach using the model of severe Ova-induced intestinal allergy (Burggraf et al., *in revision*, see 3.3.1). Mice were treated with equimolar amounts of Ova (10 µg), rflaA (6.9 µg), rflaA plus rOva (6.9 µg + 10 µg), or rflaA:Ova (16.9 µg) twice by i.p.-injection, then sensitized to Ova adsorbed to aluminium hydroxide (twice by i.p.-injection), and finally challenged for 5.5 to 8 days with Ova-containing food pellets. During Ova-pellet challenge mice were monitored for signs of illness, weight loss, core body temperature, and food uptake on a daily basis.

To simplify group nomenclature treatment groups were abbreviated by including the construct used for prophylactic vaccination, the sensitization, and the challenge in chronological order (see 3.3.2). Meaning that in the rflaA:Ova -> Ova/A -> Ova group animals were first vaccinated with rflaA:Ova, then sensitized to Ova with alum, and finally challenged with Ova-containing food-pellets.

4.3.2.1 Prophylactic vaccination with rflaA:Ova protects against intestinal allergy

Allergic positive control groups (PBS -> Ova/A -> Ova) as well as Ova- (Ova -> Ova/A -> Ova), rflaA- (rflaA -> Ova/A -> Ova), and rflaA plus Ova- (rflaA + Ova -> Ova/A -> Ova) vaccinated groups showed signs of illness such as ruffed fur, reduced mobility, and increasing softness of faeces during the time of Ova-pellet challenge. Hereby, the consistency of the faeces ranged from soft, over red brown faeces (probably containing blood) to white mucus-like faeces (Figure 48 and Figure 49). Noteworthy, no comparable signs of illness were observed for rflaA:Ova vaccinated mice (rflaA:Ova -> Ova/A -> Ova) or mice receiving normal food (PBS -> PBS -> NF, Ova -> Ova/A -> NF, Figure 48).

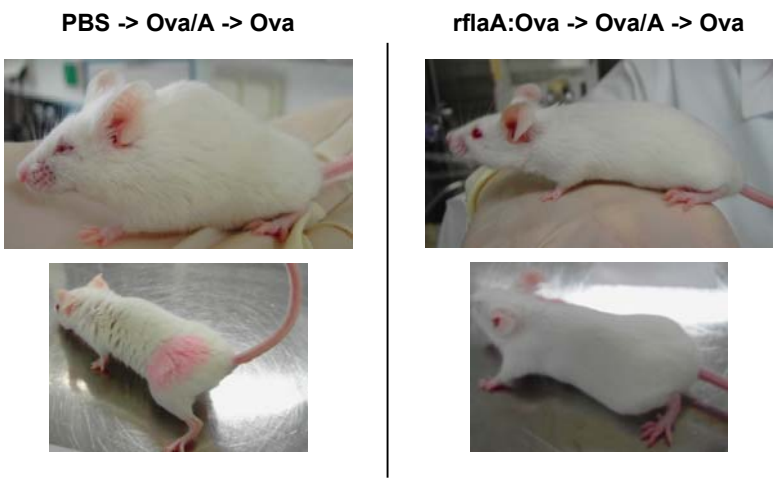


Figure 48: rflaA:Ova-vaccinated animals show no signs of illness. Phenotype of positive control (PBS –> Ova/A -> Ova) and rflaA:Ova- (rflaA:Ova –> Ova/A -> Ova) vaccinated groups. Both groups were continuously challenged with Ova-containing food pellets for 6.5 days. Photos were taken between day 3.5 and 6.5 of Ova-pellet challenge. Ova-, rflaA-, and rflaA plus Ova-vaccinated groups showed similar symptoms as the depicted positive control group. Group nomenclature: (vaccination -> sensitization -> challenge).

In order to quantify the severity of disease during Ova-pellet challenge a symptom score (ranging from 0 to 5) was established. Hereby, phenotype and behaviour were determined for every mouse on a daily base. Subsequently, softness of faeces as well as phenotypic signs of illness (ruffed fur, reduced mobility) for each mouse were used to calculate partial scores which were summed up to obtain the overall symptom score (Figure 49).

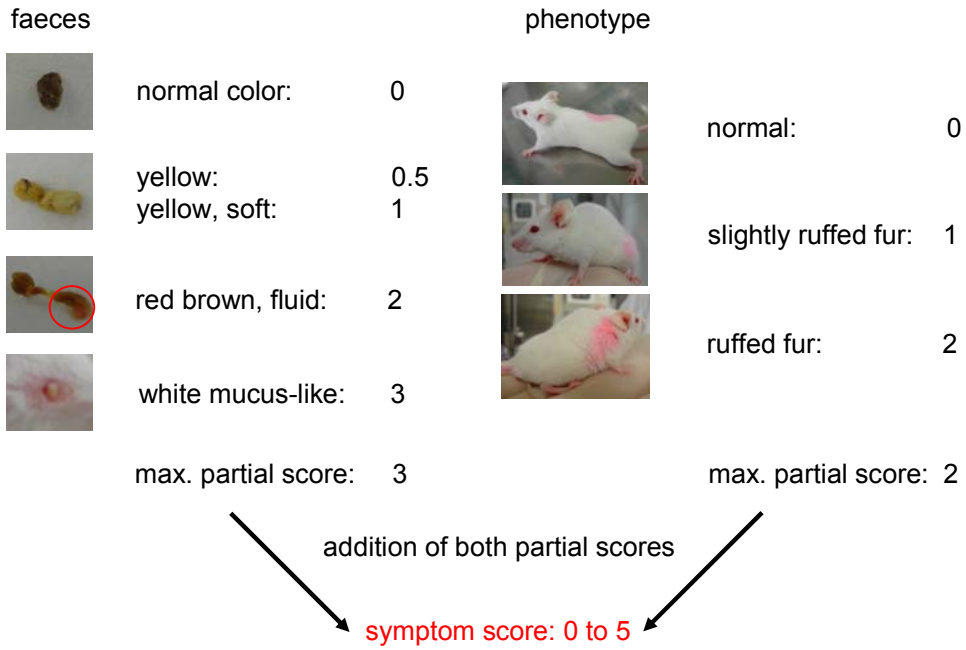


Figure 49: Symptom score used for evaluation of mouse disease symptoms. For determination of symptom scores individual mice were assessed for softness of faeces and phenotype (ruffed fur, reduced mobility). Partial scores were summed up to obtain the overall symptom score.

Whereas symptom scores for non-vaccinated positive control (PBS -> Ova/A -> Ova), Ova- (Ova -> Ova/A -> Ova), rflaA- (rflaA -> Ova/A -> Ova), and rflaA plus Ova- (rflaA + Ova -> Ova/A -> Ova) vaccinated groups steadily increased with each day of Ova-pellet challenge (resulting in a mean score of 4 on day 6.5 of Ova-pellet challenge), symptom scores for rflaA:Ova-vaccinated animals remained constant at a score of about 1 during the whole time of challenge (Figure 50). Therefore, on day 4.5 to 6.5 of Ova-pellet challenge during the most acute phase of the disease, fusion protein vaccinated mice displayed a mean symptom score that was 4-times lower than either non-vaccinated (PBS -> Ova/A -> Ova) or Ova-, rflaA-, or rflaA plus Ova- vaccinated mice (Figure 50). No significant signs of disease were observed for non-challenged groups receiving normal food (PBS -> PBS -> NF, Ova -> Ova/A -> NF, Figure 50).

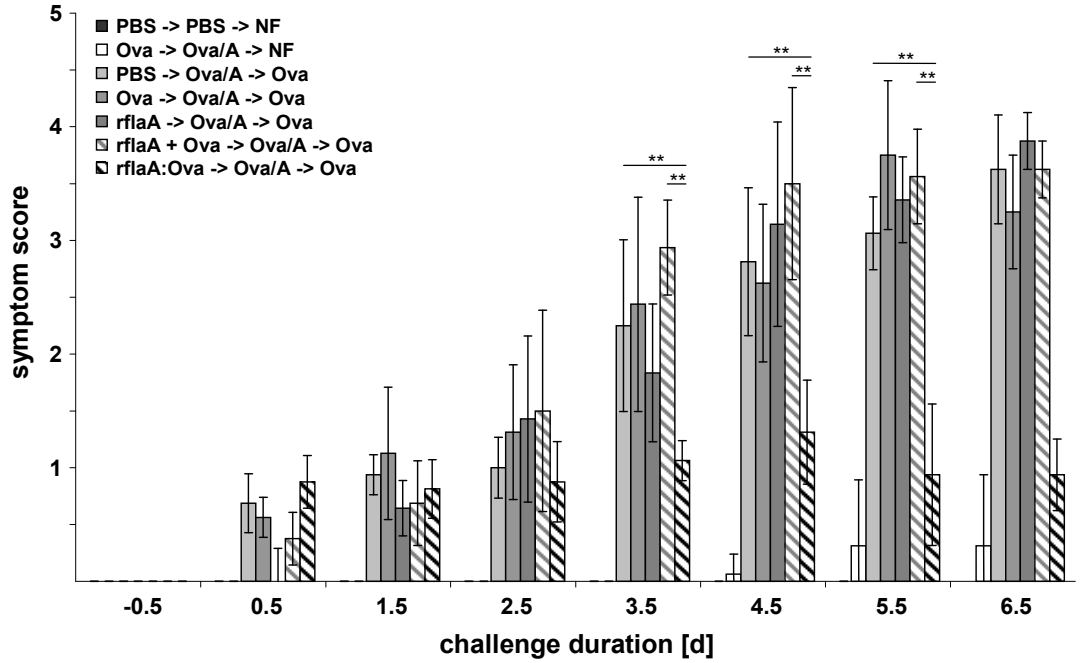


Figure 50: Prophylactic vaccination with rflaA:Ova strongly reduces disease symptoms. Control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were continuously challenged with either Ova-containing pellets (Ova) or normal pellets (NF) for 6.5 days. Disease symptoms were evaluated for each mouse on a daily base. Mean symptom scores were calculated for each group (n = 8 mice per group). Group nomenclature: (vaccination -> sensitization -> challenge). Statisical comparison was performed using the Wilcoxon signed-rank test.

To investigate the influence of vaccination and Ova-pellet challenge on food uptake, mean Ova-pellet uptake values per cage (4 mice per cage) were determined for the different treatment groups. In allergic mice (PBS -> Ova/A -> Ova) and Ova- (Ova -> Ova/A -> Ova), rflaA- (rflaA -> Ova/A -> Ova), and rflaA plus Ova- (rflaA + Ova -> Ova/A -> Ova) vaccinated groups Ova-pellet challenge resulted in a drop of mean Ova-pellet uptake from 2.5 to 3 g per

mouse (at the beginning of challenge) to a minimum of about 1.0 g observed on days 4.5 and 5.5 of Ova-pellet challenge (Figure 51). For rflaA:Ova-vaccinated animals this drop in pellet uptake was less pronounced, with a mean food uptake of 3 to 3.5 g per mouse (at the beginning of challenge) which was reduced to approximately 2 g per mouse on days 4.5 and 5.5 of Ova-pellet challenge. Nevertheless, food uptake for each day of challenge was higher for rflaA:Ova-vaccinated mice than for the respective control groups (PBS -> Ova/A -> Ova, Ova -> Ova/A -> Ova, rflaA -> Ova/A -> Ova, and rflaA + Ova -> Ova/A -> Ova, respectively Figure 51). Non-challenged groups receiving normal food (PBS -> PBS -> NF, Ova -> Ova/A -> NF) displayed a mean pellet uptake between 2.5 and 3.7 g per mouse and day (Figure 51). In these groups no minima of food uptake on day 4.5 and 5.5 of Ova-pellet challenge were detected. Since the Ova-pellets used contained approximately 30% ovalbumin it was possible to calculate the amount of allergen taken up. Daily Ova-uptake ranged from 1 g per mouse and day on day 0.5 to approximately 0.3 g on days 4.5 and 5.5 of Ova-pellet challenge (Figure 51).

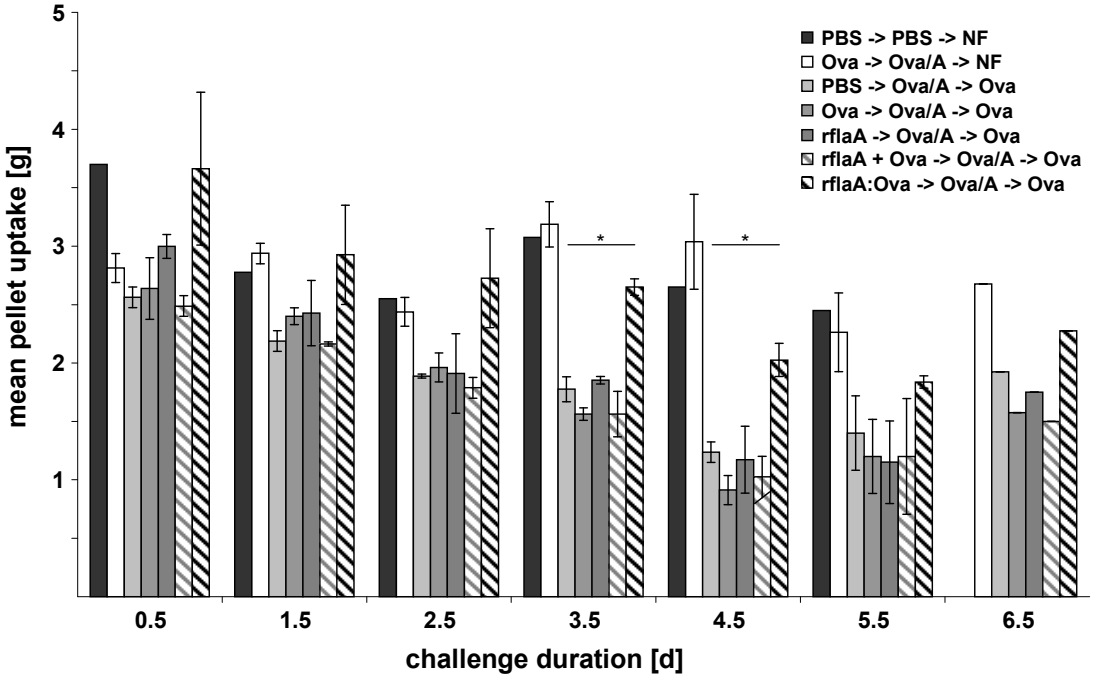


Figure 51: rflaA:Ova-vaccinated mice show a higher food uptake than control groups. Control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were continuously challenged with either Ova-containing pellets (Ova) or normal pellets (NF) for 6.5 days. Pellets were weighted every day and mean pellet uptake values per mouse and day were calculated for each group (n = 8 mice per group, 2 cages with 4 mice each). Group nomenclature: (vaccination -> sensitization -> challenge).

4.3.2.2 *Vaccination with rflaA:Ova protects against weight loss and drop in core body temperature*

When monitoring body weights for the different groups, rflaA:Ova-vaccinated animals increased in body weight by 2 to 3% after 6.5 day of challenge (compared to body weights before challenge, approximately 21 to 23 g per mouse), whereas mean body weights per group were reduced by 5 to 9% in the respective control groups (PBS -> Ova/A -> Ova, Ova -> Ova/A -> Ova, rflaA -> Ova/A -> Ova, and rflaA + Ova -> Ova/A -> Ova, respectively Figure 52). Hereby, animals vaccinated with the mixture of rflaA and Ova (rflaA + Ova -> Ova) displayed the highest weight loss. In non-challenged groups receiving normal food (PBS -> PBS -> NF, Ova -> Ova/A -> NF) mean body weight remained constant (Figure 52).

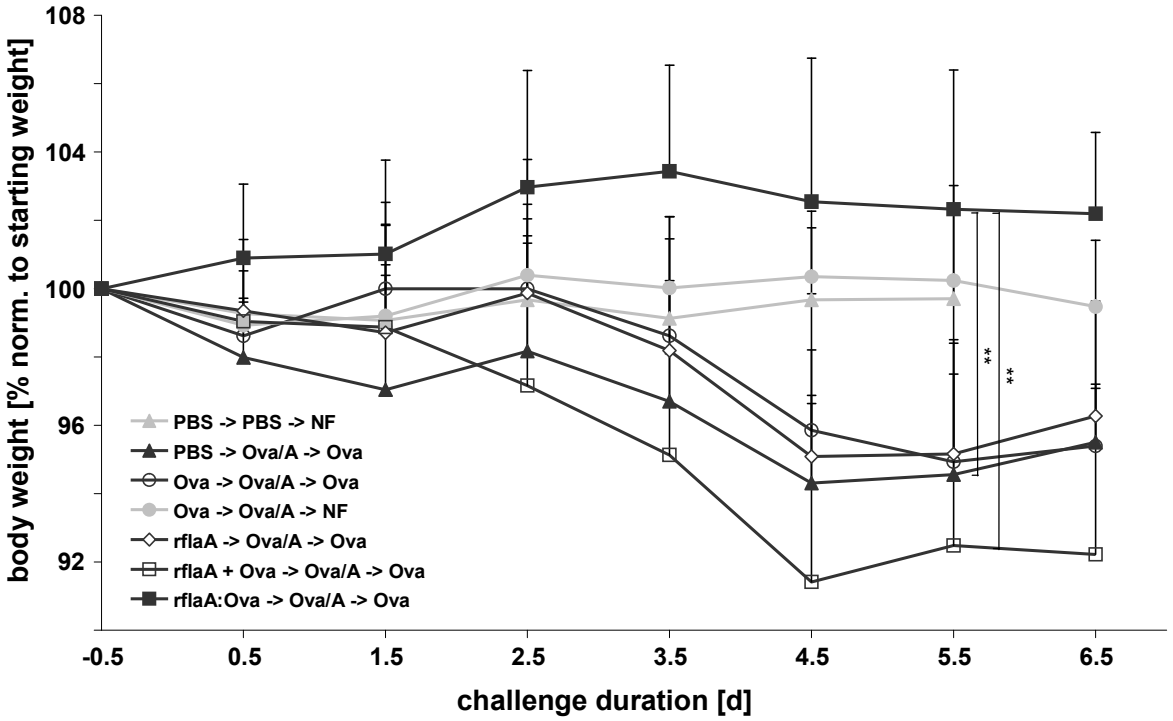


Figure 52: Prophylactic vaccination with rflaA:Ova protects against weight loss. Control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were continuously challenged with either Ova-containing pellets (Ova) or normal pellets (NF) for 6.5 days. Body weight for every mouse was determined, normalized to the individual starting weight, and mean normalized body weights were calculated for each day and group (n = 8 mice per group). Group nomenclature: (vaccination -> sensitization -> challenge).

Moreover, body temperatures for each mouse were determined rectally on a daily base. A drop in core body temperature was observed in the Ova-induced intestinal allergy model (PBS -> Ova/A -> Ova, Figure 53). This temperature drop was shown to first occure between days 3.5 and 4.5 of Ova-pellet challenge in Ova -> Ova/A -> Ova, rflaA -> Ova/A -> Ova, and rflaA + Ova -> Ova/A -> Ova groups (Figure 53). In these groups mean core body temperatures were reduced by 2 to 4°C (Figure 53). In accordance with the results obtained

for mouse body weights (Figure 52) the observed drop in core body temperature was most pronounced for mice vaccinated with a mixture of rflaA and rOva (rflaA + Ova -> Ova/A -> Ova, Figure 53). In contrast to this, both rflaA:Ova-vaccinated mice and control groups receiving normal food (PBS -> PBS -> NF, Ova -> Ova/A -> NF) displayed stable core body temperatures ranging from 37 to 38°C (Figure 53).

Taken together these results clearly show that prophylactic vaccination with rflaA:Ova, but not rflaA and rOva either provided alone or as a mixture was sufficient to prevent the establishment of intestinal allergy in the used mouse model.

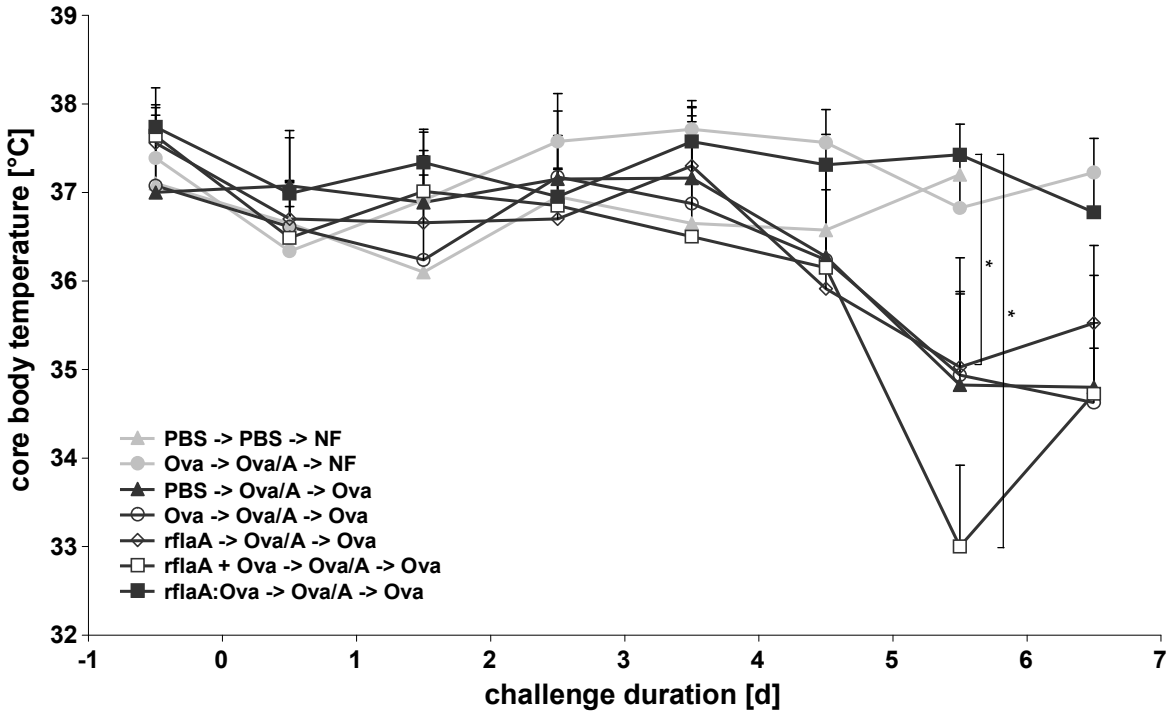


Figure 53: Prophylactic vaccination with rflaA:Ova protects against drop in core body temperature. Control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were continuously challenged with either Ova-containing pellets (Ova) or normal pellets (NF) for 6.5 days. Core body temperatures for every mouse were determined rectally and mean body temperatures were calculated for each day and group (n = 8 mice per group). Group nomenclature: (vaccination -> sensitization -> challenge).

4.3.2.3 Vaccination does not affect T cell-proliferation upon re-stimulation

To investigate whether prophylactic treatment might influence T cell priming and responses upon restimulation, CD4 T cells were isolated from spleens and mesenteric lymph nodes (MLN) of vaccinated and control groups (see 3.3.4). The purified T cells were co-cultured with syngenic APC and restimulated with Ova (Repository figure 1 and Repository figure 2).
Due to unkown reasons unstimulated co-cultures of both splenic and mesenterial lymph node T cells displayed rather high basic proliferation rates (Repository figure 1 and Repository figure 2). Therefore, when comparing T cell proliferation between the different vaccination groups no differences in Ova-induced proliferation were detectable (Repository figure 1 and Repository figure 2).

4.3.2.4 Prophylactic vaccination does not influence T_{reg} frequency

To investigate whether the preventive effect of rflaA:Ova might be due to an increased induction of regulatory T cells, $CD4^+CD25^+$Fox p 3^+ T_{regs} in spleen and MLN were quantified by flow cytometry (Figure 54 and Figure 55). Frequencies of T_{reg} in mesenteric lymph nodes on day 5.5 did not reveal pronounced differences between treatment groups. However, slightly elevated levels of regulatory T cells were detected in non-challenged (PBS -> PBS -> NF), rflaA-, rflaA plus Ova-, and rflaA:Ova-vaccinated mice compared to untreated (PBS -> Ova/A -> Ova), Ova treated but non-challenged (Ova -> Ova/A -> NF), and Ova treated (Ova -> Ova/A -> Ova) animals (Figure 54). In contrast to this all groups displayed comparable T_{reg} frequencies on day 6.5 of Ova-pellet challenge (Figure 54).

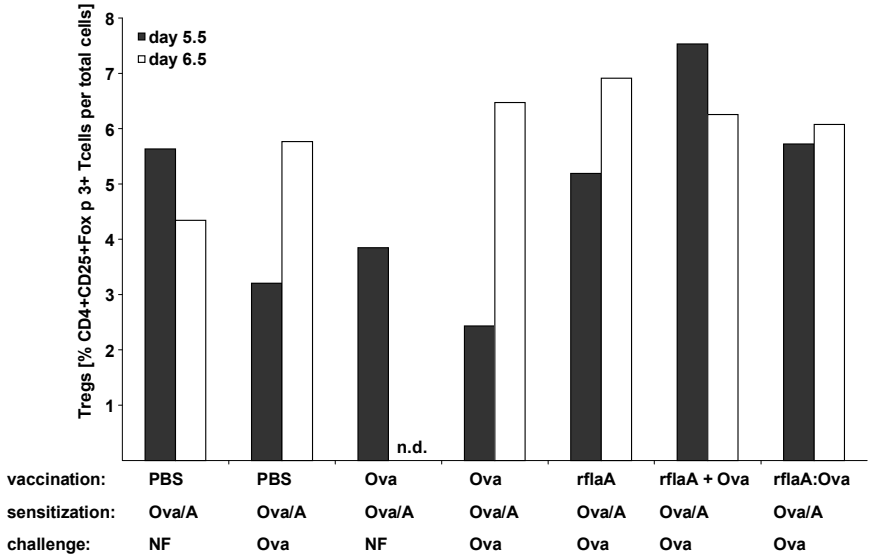


Figure 54: Prophylactic vaccination with rflaA:Ova does not increase MLN T_{reg} frequencies. On day 5.5 (black) and 6.5 (white) of Ova-pellet challenge MLN of 4 animals per group were dissected under sterile conditions, crushed, filtered, and pooled. After lysis of red blood cells 10^6 cells were stained for $CD4^+CD25^+$Fox p 3^+ T_{regs} using the T_{reg} staining kit. Cells were gated on CD4 T cells and the frequencies of $CD4^+CD25^+$Fox p 3^+ T_{regs} were determined by analyzing 10^5 T cells. n.d .not determined.

In spleen samples obtained on day 6.5 rflaA- and rflaA plus Ova-vaccinated animals displayed slightly increased levels of $CD4^+CD25^+$Fox p 3^+ regulatory T cells, whereas again on day 5.5 of Ova-pellet challenge no differences in T_{reg} frequencies were detectable (Figure 55).

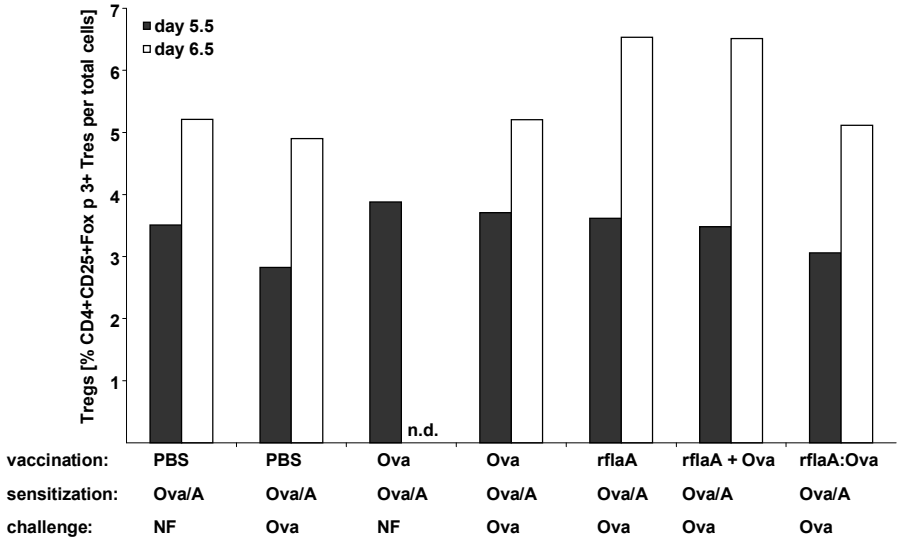


Figure 55: Prophylactic vaccination with rflaA:Ova does not increase splenic T_{reg} frequencies. On day 5.5 (black) and 6.5 (white) of Ova-pellet challenge spleens of 4 animals per group were dissected under sterile conditions, crushed, filtered, and pooled. After lysis of red blood cells 10^6 cells were stained for $CD4^+CD25^+$Fox p 3^+ T_{regs} using the T_{reg} staining kit. Cells were gated on CD4 T cells and the frequencies of $CD4^+CD25^+$Fox p 3^+ T_{regs} were determined by analyzing 10^5 T cells. n.d .not determined.

4.3.2.5 Flow cytometric analyses reveal a reduced T cell activation upon rflaA:Ova vaccination

To further characterize the immune responses upon Ova-pellet challenge in the different treatment groups, the activation status of B cells, T cells, and DC was investigated by flow cytometry (Figure 56 and Figure 57). In order to evaluate the effects of vaccination with the different constructs on intestinal allergy all groups were compared to the non-vaccinated allergy positive control group (PBS -> Ova/A -> Ova).

On splenic cells obtained on day 5.5 of Ova-pellet challenge only slight differences in marker expression were detected on CD19 B cell and CD11 dendritic cell subsets (Repository figure 5). Hereby, a slight down-regulation of CD86 and MHC II expression on B cells was observed in non-challenged (either non-vaccinated or Ova-vaccinated groups) and rflaA:Ova-vaccinated animals (Repository figure 5). More striking differences were observed for CD4 and CD8 T cell subsets. When checking for T cell activation during the acute phase of the disease (day 5.5) non-challenged (Ova ->Ova/A -> NF), rflaA-, and rflaA plus Ova-vaccinated groups displayed elevated levels of CD62-L expression on both CD4 and CD8 T cell subsets compared to the untreated control group (PBS -> Ova/A -> Ova). CD62-L levels were further increased in rflaA:Ova vaccinated animals (Figure 56). Since CD62-L is a selectin involved in T cell homing which is down-regulated upon T cell activation these results suggest that mice vaccinated with the fusion protein have a reduced T cell activation compared to the other groups. In line with these results, a down-regulation of PD-1 expression, an ITIM-containing immuno receptor on CD4 T cells was observed in rflaA- and rflaA plus Ova-vaccinated animals, which was less pronounced in rflaA:Ova-treated animals (Figure 56).

On spleen cells obtained on day 6.5, according to day 5.5 of Ova-pellet challenge higher levels of CD62-L expression were recorded for rflaA-, rflaA plus Ova-, and rflaA:Ova-vaccinated animals (Figure 56 B). Hereby, levels of CD62-L were highest in rflaA- and rflaA plus Ova-vaccinated groups. Moreover, a reduced expression of PD-1 in rflaA-, rflaA plus Ova-, and rflaA:Ova-vaccinated animals was recorded compared to non-vaccinated animals. PD-1 expression was not different for the other treatment groups (Figure 56 B). In accordance with the results obtained on day 5.5 B cells and DC demonstrated only very slight changes in surface marker expression (Repository figure 5). As described above on day 5.5 for B cells a down-regulation of CD86 and MHC II was observed in non-challenged (PBS -> Ova/A -> NF), rflaA plus Ova-, and rflaA:Ova-vaccinated animals (Repository figure 6).

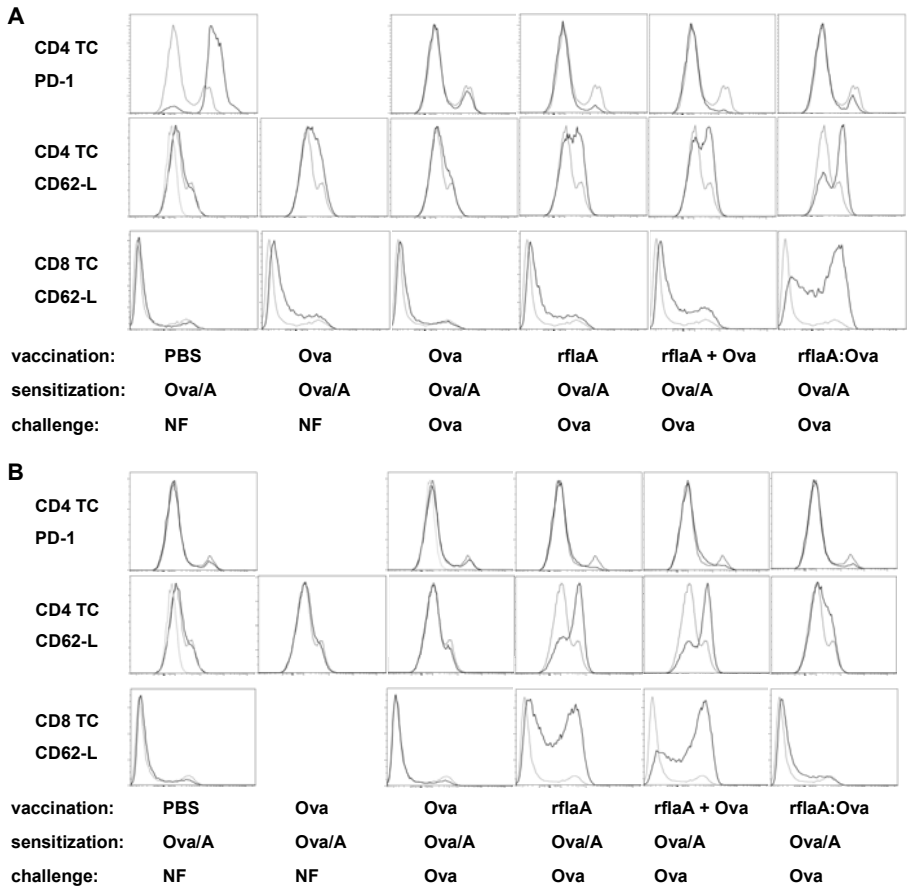


Figure 56: Prophylactic vaccination with rflaA:Ova prevents splenic TC activation. On day 5.5 (A) and 6.5 (B) of Ova-pellet challenge animals were sacrificed, spleens were prepared, and four animals per group were pooled. Subsequently, red blood cells were lysed and cells were stained for the indicated lineage and cell surface markers. Expression levels were determined for gated (as indicated) cell populations via flow cytometry and compared to the PBS -> Ova (allergic positive control, grey) group.

In samples obtained from mesenteric lymph nodes on day 5.5 CD62-L expression on CD4 T cells was strongly enhanced in non-challenged (PBS -> Ova/A -> NF, Ova -> Ova/A -> NF) and Ova-treated animals, whereas in rflaA plus Ova and rflaA:Ova-vaccinated animals the up-regulation of CD62-L was less pronounced but still clearly detectable. In rflaA-treated animals no differences in CD62-L expression were detected (Figure 57 A). Furthermore, no differences were detected for CD69 expression in the investigated treatment groups (data not shown). On CD8 T cells CD62-L expression was reduced in rflaA-, rflaA plus Ova-, and rflaA:Ova-vaccinated groups, but not in animals receiving normal food (PBS -> Ova/A -> NF, Ova -> Ova/A -> NF, Figure 57 A). On the CD19 B cell subset up-regulation of CD80 was detected in non-challenged groups (PBS -> Ova/A -> NF, Ova -> Ova/A -> NF) compared to non-vaccinated animals (PBS -> Ova/A -> Ova). In contrast to this rflaA-, rflaA plus Ova-, and rflaA:Ova-treated animals demonstrated a reduced CD80 expression (Repository figure 7). A similar expression pattern was detected for CD86 where expression levels were reduced in rflaA- and rflaA:Ova-treated groups, whereas CD69 expression was only reduced

in rflaA-vaccinated animals (Repository figure 7). A slight increase of MHC I expression was detected on both B cells and dendritic cells obtained from rflaA- and rflaA:Ova-vaccinated animals (Repository figure 7). On CD11c DC a upregulation of the activation marker CD40 was detected in rflaA- and rflaA:Ova-vaccinated animals, whereas CD86 was down-regulated in rflaA-, rflaA plus Ova-, and rflaA:Ova-treated animals (Repository figure 7).

On day 6.5 of Ova-pellet challenge cells from MLN isolates showed higher CD62-L expression on CD4 and CD8 T cells in non-challenged, rflaA- (only on $CD4^+$ T cells), and rflaA plus Ova-vaccinated groups, whereas for rflaA:Ova-vaccinated animals no difference in CD62-L expression was detectable compared to non-vaccinated animals (Figure 57). On CD19 B cells a higher expression of the activation markers CD69 and CD80 was detected in non-challenged animals (PBS -> Ova/A -> NF) compared to all other treatment groups (Repository figure 8), indicating that B cells were not fully activated in the different vaccination groups. Furthermore, B cells displayed a down-regulation of MHC II expression on rflaA- and rflaA plus Ova-vaccinated animals, which was less pronounced in rflaA:Ova-vaccinated animals (Repository figure 8). For CD11c DC no remarkable expression differences were detected for the investigated markers (Repository figure 8).

In summary prophylactic vaccination with rflaA:Ova reduced splenic T cell activation (as determined by high expression levels of CD62-L) on days 4.5 and 5.5 of Ova-pellet challenge compared to the other treatment groups. In mesenteric lymph nodes a similar reduced T cell activation was only observed on CD4 T cells on day 5.5, whereas on day 6.5 no differences in T cell activation were detected.

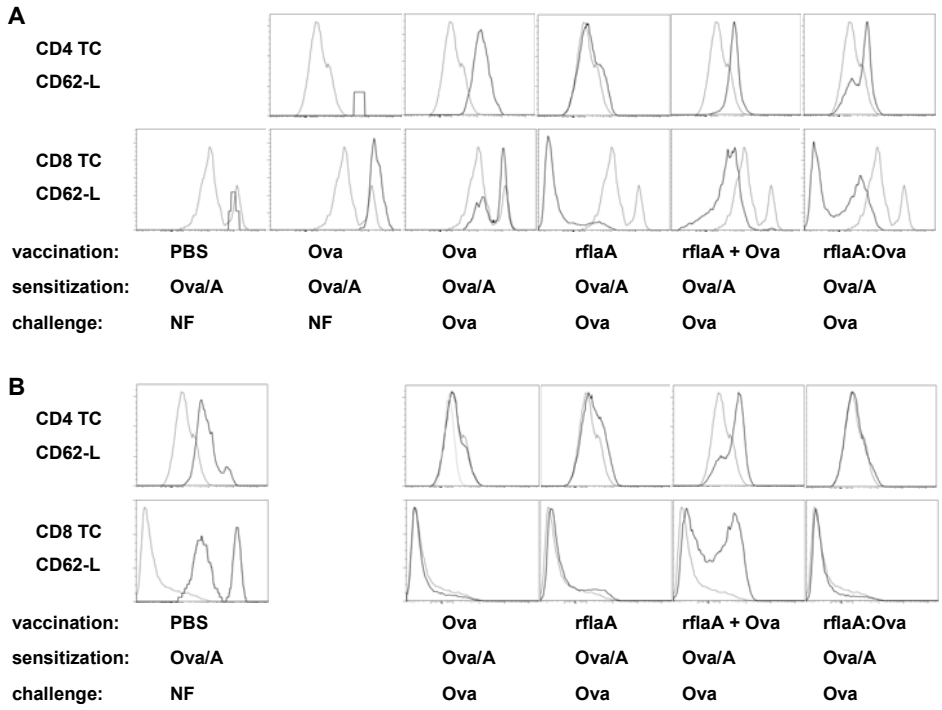


Figure 57: Prophylactic vaccination with rflaA:Ova and rflaA plus Ova prevents MLN TC activation. On day 5.5 (A) and 6.5 (B) of Ova-pellet challenge animals were sacrificed, mesenteric lymph nodes were prepared, and three animals per group were pooled. Subsequently, red blood cells were lysed and cells were stained for the indicated lineage and cell surface markers. Expression levels were determined for gated (as indicated) cell populations via flow cytometry and compared to the PBS -> Ova (allergic positive control, grey) group.

4.3.2.6 Prophylactic vaccination with rflaA:Ova suppresses Th2 cytokines in intestinal homogenates

When homogenates prepared form intestinal samples (see 3.3.9) were tested for cytokine production by multiplex ELISA rflaA-, rflaA plus Ova-, and rflaA:Ova-vaccinated animals displayed higher levels of IL-1α and MCP-1 than Ova-vaccinated or non-vaccinated groups (Figure 58). In agreement with the high symptome scores, distinct weigth loss, and temperature drop observed in rflaA plus Ova-vaccinated animals (see 4.3.2.1 and 4.3.2.2) high levels of Th2 cytokines IL-4 and IL-13 were detected in corresponding intestinal homogenates (Figure 58). In contrast to this, mice vaccinated with the fusion protein displayed lower median levels of IL-4 and IL-13, comparable to unchallenged animals (Figure 58). Interestingly, median IL-5 levels were not different between the treatment groups (Figure 58). Moreover, in accordance with the reduced symptoms in the rflaA:Ova-vaccinated group four out of eight animals vaccinated with the fusion construct had strongly increased intestinal levels of the immunosuppressive cytokine IL-10, whereras no differences were observed for the other groups (Figure 58). Interestingly, only rflaA:Ova-vaccinated animals had increased levels of IL-23 in intestinal homogenates which did not result in increased IL-17 production (Figure 58). Simillarly, IFN-γ production was solely detected in three out of 8 mice in the rflaA:Ova-vaccinated group (Figure 58). IL-18 production was only detected in either non-challenged (PBS -> PBS -> NF, Ova -> Ova/A -> NF) or rflaA:Ova-vaccinated animals (Figure 58). No differences between the different treatment groups were detected for GM-CSF, IL-6 (Figure 58), IL-2, and IL-22 (data not shown). Moreover, cytokines IL-17 and TNF-α were undetectable (data not shown).

In summary analysis of cytokine levels in intestinal homogenates revealed lower Th2-cytokine production in fusion protein vaccinated mice compared to either unvaccinated or rflaA plus Ova-vaccinated animals. Moreover, four out of eight mice treated with the fusion protein displayed detectable levels of IL-10 in intestinal homogenates. These results are in accordance with the strongly reduced symptoms in rflaA:Ova-vaccinated animals.

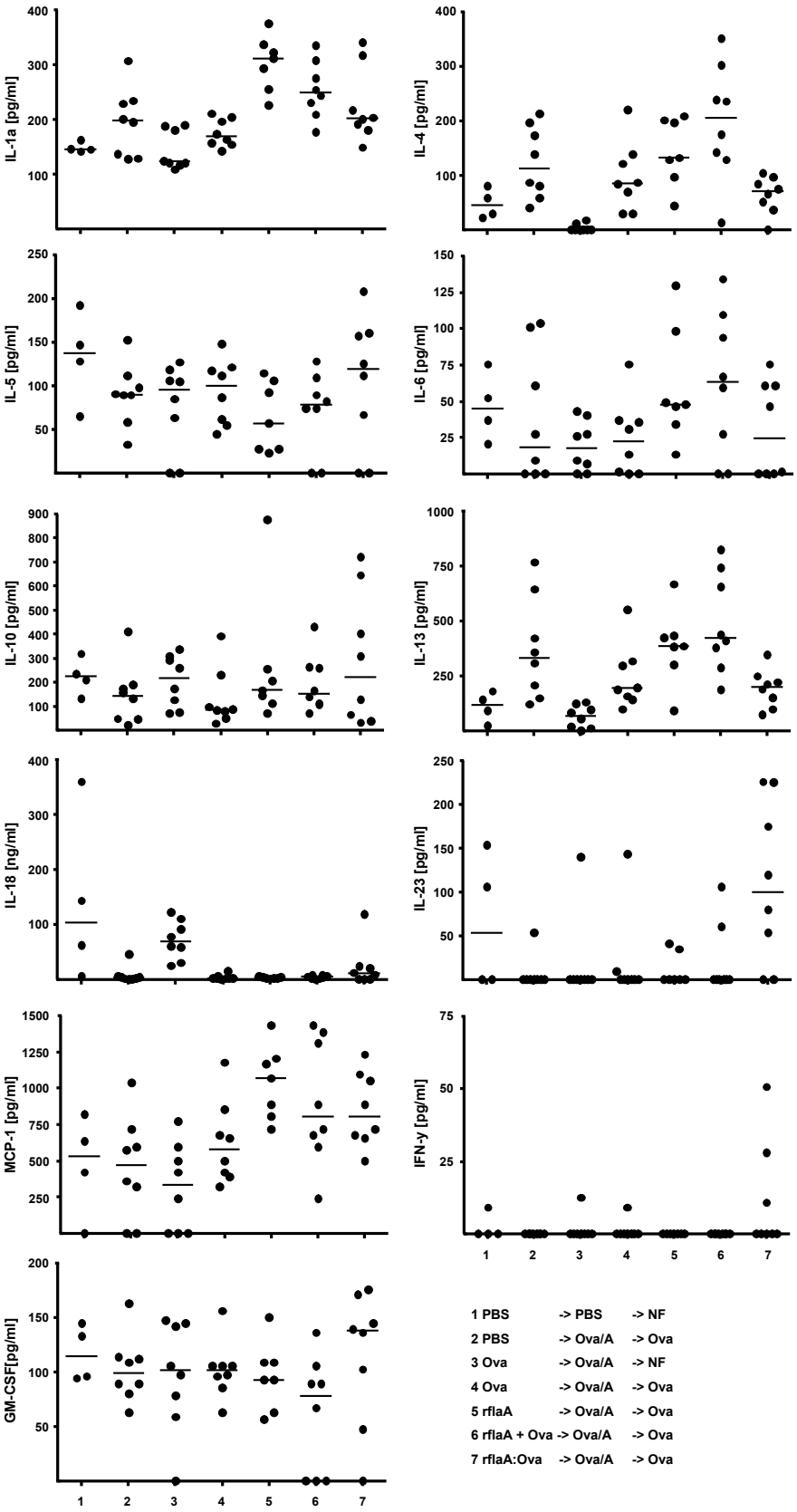


Figure 58: Determination of cytokines in intestinal homogenates. Control (PBS ->PBS -> NF) and Ova-sensitized mice (all other groups) were continuosly challenged with either Ova-containing pellets (Ova) or normal pellets (NF) for 5.5 or 6.5 days (4 mice per time point). Intestines were prepared and total proteins isolated. Median cytokine levels were detected via multiplex ELISA (n = 8 mice per group). Group nomenclature: (vaccination -> sensitization -> challenge).

4.3.2.7 Prophylactic vaccination with rflaA:Ova induces Ova-specific IgG2a- and suppresses IgE production

To further characterize the type of immune response in the different vaccination groups sera were checked for the induction of Ova-specific IgG1, IgG2a, and IgE antibodies (Figure 59 to Figure 62). When testing for Ova-specific IgG1 induction in the different vaccination and control groups, one week after the first immunization rflaA plus Ova-vaccinated animals displayed with a mean level of approximately 120 µg/ml IgG1 slightly higher Ova-specific IgG1 levels than the other groups (between 0 and 40 µg/ml, Figure 59 A), whereas no differences between the different treatment groups were detectable one week after the second immunization or at the end of Ova-pellet challenge (Figure 59 B and C). As expected, both non-vaccinated and rflaA-vaccinated animals showed a delayed induction of Ova-specific antibodies compared to the groups that were treated with either Ova alone, mixed with rflaA, or covalently fused to it (Figure 59).

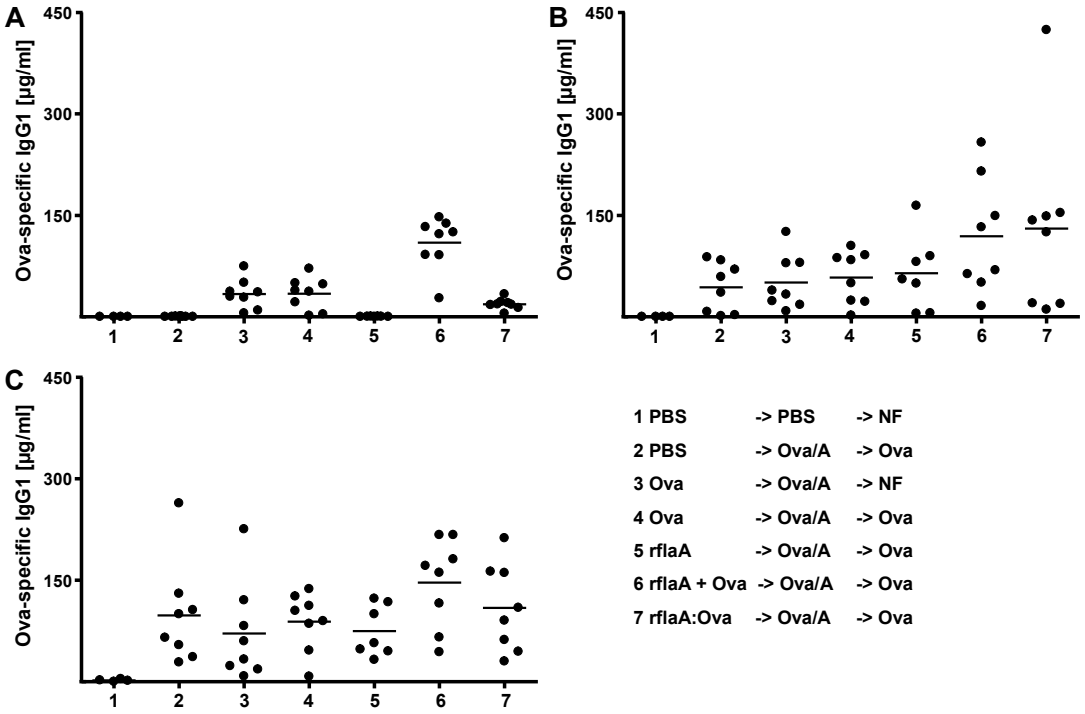


Figure 59: Prophylactic vaccination with rflaA:Ova does not induce IgG1 production. Sera of control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were collected one week after the first immunization (A), one week after the second immunization (B), and on the final day of Ova pellet challenge (day 5.5 or 6.5, C). Sera were checked for levels of Ova-specific IgG1 antibodies by ELISA (n = 8 mice per group). Group nomenclature: (vaccination -> sensitization -> challenge).

In contrast to this, rflaA:Ova-vaccinated animals displayed high levels of Ova-specific IgG2a antibodies, with mean levels ranging from 2000 to 3800 ng/ml, whereas only low levels of Ova-specific IgG2a antibodies (below 500 ng/ml) were detected in the other treatment

groups (Figure 60). These differences were already detectable one week after the first sensitization (mean IgG2a level: 2000 ng/ml, Figure 60 A), but with a mean value of approximately 3800 ng/ml even more pronounced one week after the second immunization (Figure 60 B). After Ova-pellet challenge mean IgG2a levels were slightly lower compared to one week after the second immunization (2000 vs. 3800 ng/ml). However, individual mice displayed a much more homogenous distribution of IgG2a levels (Figure 60 C). Hereby, every mouse treated with the fusion protein had higher Ova-specific IgG2a levels compared to the other groups.

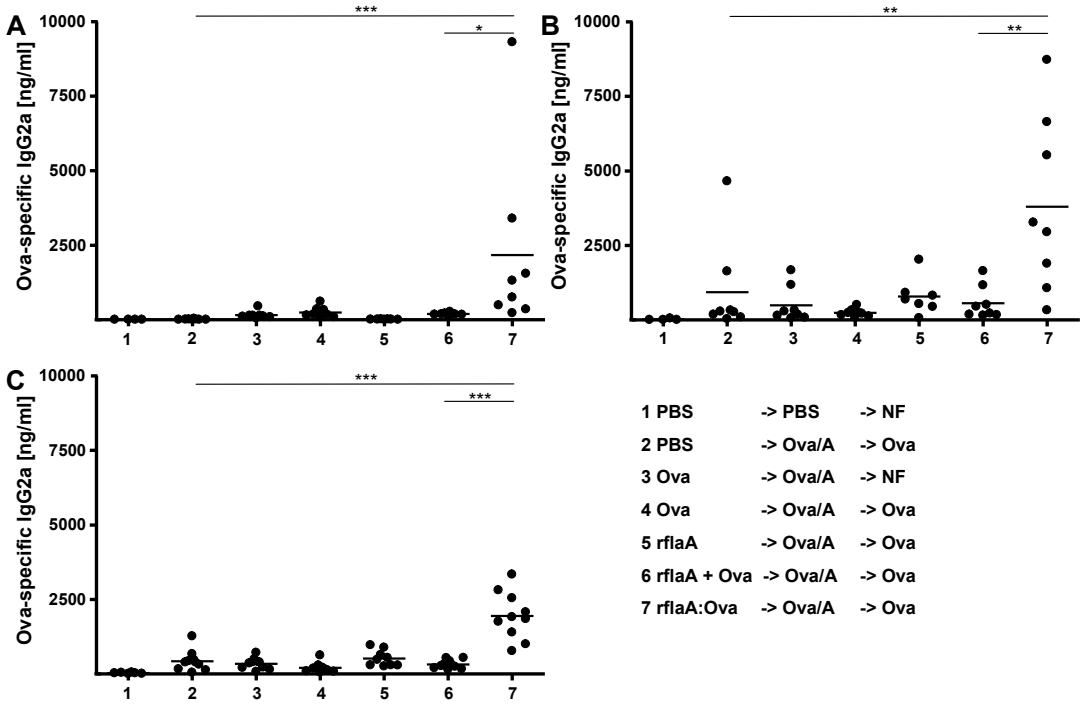


Figure 60: Prophylactic vaccination with rflaA:Ova induces IgG2a production. Sera of control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were collected one week after the first immunization (A), one week after the second immunization (B), and on the final day of Ova pellet challenge (day 5.5 or 6.5, C). Sera were checked for levels of Ova-specific IgG2a antibodies by ELISA (n = 8 mice per group). Group nomenclature: (vaccination -> sensitization -> challenge).

In contrast to this for Ova-specific IgE-levels a constant increase over time was detected in sera of non-vaccinated, Ova-, rflaA-, and rflaA plus Ova-vaccinated groups (Figure 61). Hereby, detectable levels of Ova-specific IgE were observed in most groups one week after the second immunization, ranging from 125 (Ova -> Ova/A -> Ova) to 160 ng/ml (rflaA + Ova -> Ova/A -> Ova, Figure 61 B). At the end of Ova-pellet challenge IgE levels were even further increased in these groups ranging from 220 (PBS -> Ova/A -> Ova) to 350 ng/ml IgE (rflaA + Ova -> Ova/A -> Ova, Figure 61 C). Noteworthy, sera obtained from rflaA:Ova-vaccinated mice showed with 0 to 50 ng/ml strongly decreased IgE-levels at all investigated time points. At the end of Ova-pellet challenge Ova-specific IgE-levels in the sera of

rflaA:Ova-vaccinated animals were approximately ten times lower than in either non-vaccinated, Ova-, and rflaA plus Ova vaccinated animals (Figure 61). Expectedly, no Ova-specific IgE antibodies were detected in non-treated animals (PBS -> PBS -> NF).

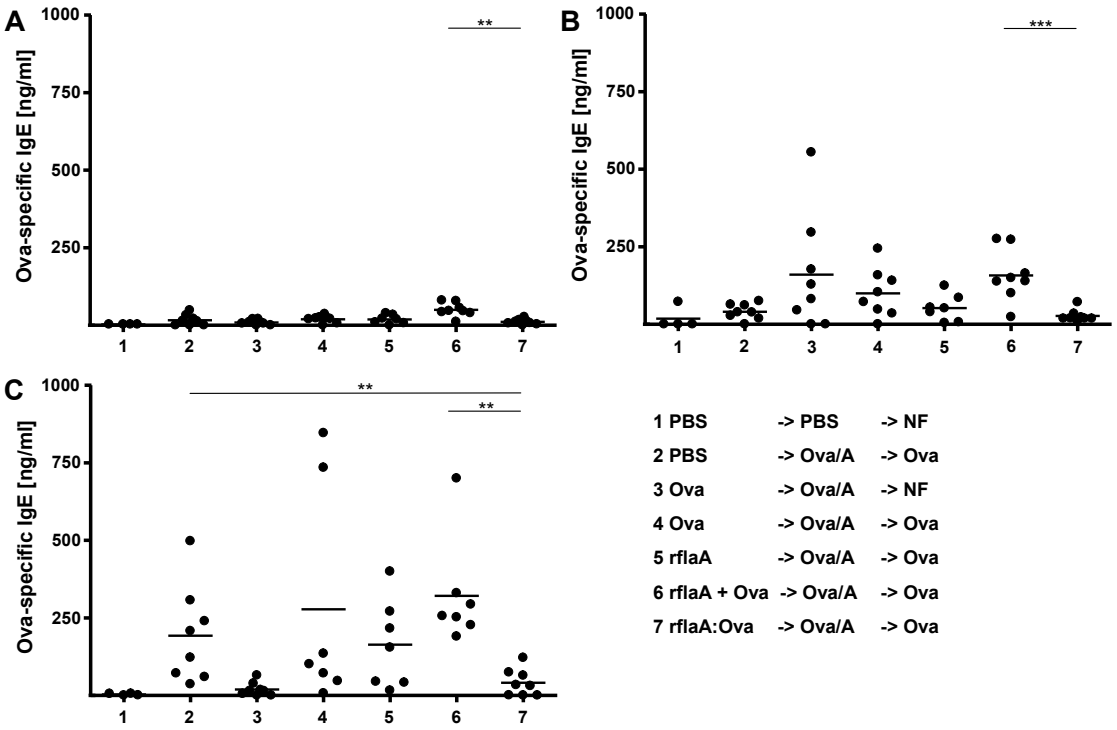


Figure 61: Prophylactic vaccination with rflaA:Ova suppresses IgE production. Sera of control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were collected one week after the first immunization (A), one week after the second immunization (B), and on the final day of Ova pellet challenge (day 5.5 or 6.5, C). Sera were checked for levels of Ova-specific IgE antibodies by ELISA (n = 8 mice per group). Group nomenclature: (vaccination -> sensitization -> challenge).

To better characterize the type of immune response induced in the different treatment groups IgG2a/IgE ratios were determined for each mouse and time point (Figure 62). Unfortunenately for some animals no IgG2a/IgE ratios could be calculated since in these animals no Ova-specific IgE could be detected (mostly in the non-treated PBS -> PBS -> NF group). Here, already one week after the first sensitization mean IgG2a/IgE ratios were at least 70-fold higher in rflaA:Ova-vaccinated animals than in all other treatment groups. Whereas Ova-vaccinated animals (both Ova -> Ova/A -> Ova and Ova -> Ova/A -> NF, mean IgG2a/Ige ration: 17) displayed slightly higher IgG2a/IgE ratios than either non-vaccinated (mean IgG2a/Ige ration: 0.65), rflaA- (ratio: 0.65), or flaA plus Ova-vaccinated (ratio: 4.9) animals one week after the first sensitization (Figure 62 A), no differences in IgG2a/IgE ratios between these groups were detectable one week after the second sensitization or at the end of Ova pellet challenge (Figure 62 B and C).

In summary, analysis of Ova-specific antibody responses showed that whereas no differences in IgG1 levels could be detected, prophylactic vaccination with rflaA:Ova resulted in a strong induction of IgG2a production while IgE production was reduced, indicating the induction of a more Th1-dominated immune response.

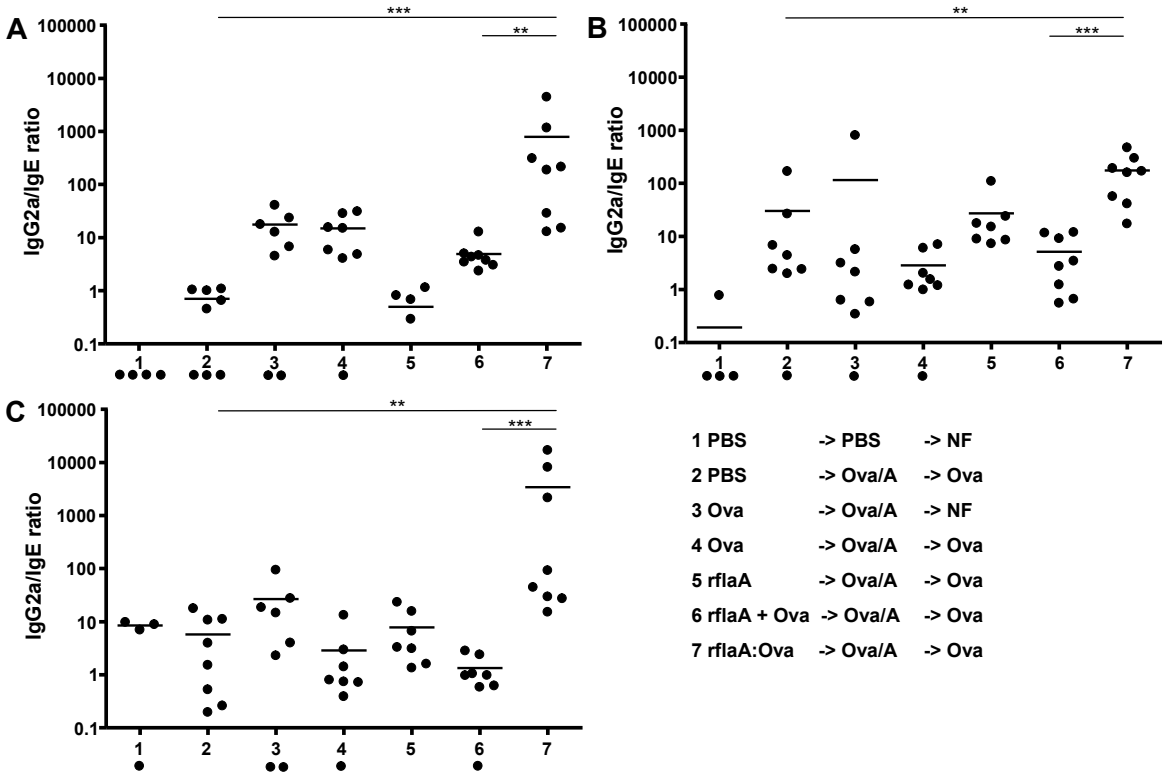


Figure 62: Prophylactic vaccination with rflaA:Ova leads to an increased IgG2a/IgE ratio. Sera of control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were collected one week after the first immunization (A), one week after the second immunization (B), and on the final day of Ova pellet challenge (day 5.5 or 6.5, C). Sera were checked for levels of Ova-specific IgG2a and IgE antibodies by ELISA, IgG2a to IgE ratios were calculated for each mouse and time point (n = 8 mice per group). Mice with non-calculable IgG2a/IgE ratios due to undetectable levels of IgE were indicated as black circles below the respective groups. Group nomenclature: (vaccination -> sensitization -> challenge).

4.3.2.8 Intranasal vaccination with rflaA:Ova protects against allergy

To evaluate the prophylactic potency of rflaA:Ova upon intranasal administration, in a preliminary study mice were either vaccinated intranasal with rflaA:Ova (three times in 3 days intervals under Ketamin/Rompun anaesthesia) or by i.p.-injection. Subsequently, symptom scores, body weights, food uptake rates, and Ova-specific antibody titers were determined and compared to either non-vaccinated or Ova-vaccinated animals (Figure 62 to Figure 65). When comparing symptom scores, intranasal vaccination proved to be as potent in preventing signs of illness as the i.p.-injection (Figure 63). Here mean symptom scores on

days 7 and 8 of Ova-pellet challenge were with 0.5 for rflaA:Ova-vaccination (either i.n or i.p.) compared to 4 to 4.4 for non-vaccinated (PBS -> Ova/A -> Ova) or Ova-vaccinated (Ova -> Ova/A -> Ova) groups 8 to 9 times lower (Figure 62 A). In accordance with these results, both intranasal and intraperitoneal treatments were sufficient to protect against Ova-pellet challenge induced weight loss (Figure 62B). Here, rflaA:Ova-vaccinated animals (either i.n or i.p.) displayed increases in body weight of 2 to 5%, whereas all other groups showed weight loss rates of 3 to 10% (Figure 62 B). Moreover, rflaA:Ova-vaccination (either i.n or i.p.) did result in a constant food uptake during the whole time of Ova-pellet challenge, whereas non-vaccinated or Ova-vaccinated animals displayed strongly reduced pellet uptakes on days 4 to 6 of challenge (reduced from 3.5 g on day 1 to 1 g on days 5 and 6, Figure 62 C).

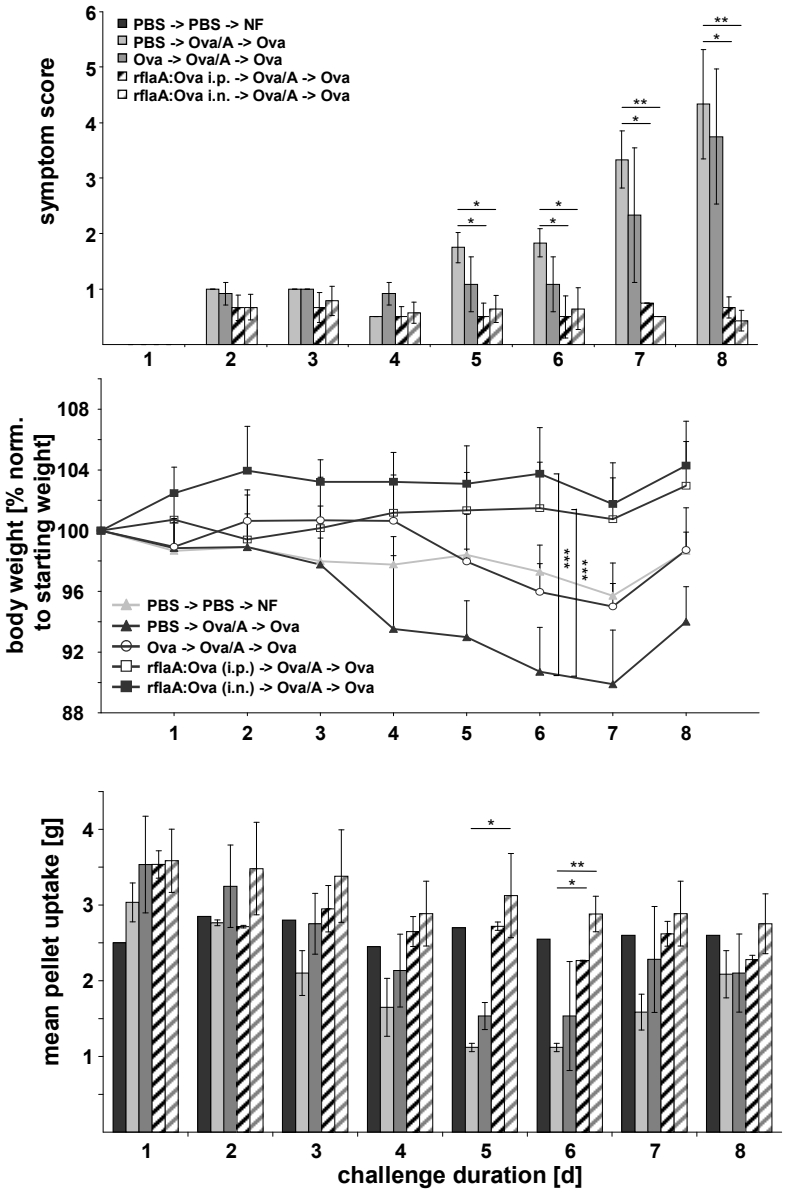


Figure 63: Intranasal administration of rflaA:Ova prevents intestinal allergy. Control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were continuously challenged with either Ova-containing pellets (Ova) or normal pellets (NF) for 8 days. Disease symptoms, body weight, and Ova-pellet uptake were evaluated for every mouse on a daily base. Mean symptom scores (A), body weights (B), and food uptake values (C) were calculated for each group (n = 6 mice per group). Group nomenclature: (vaccination -> sensitization -> challenge). Statisical comparison was either performed using the Wilcoxon signed-rank test (symptom scores) or the Bonferroni method (all other tests).

In accordance with the results presented under 4.3.2.7, investigation of Ova-specific IgG1 levels revealed no differences between the different treatment groups (data not shown). In contrast to this, Ova-specific IgG2a levels were higher in rflaA:Ova-vaccinated animals (both i.n and i.p.) than in either non-vaccinated or Ova-vaccinated groups (Figure 63). Here, compared to intranasal application i.p.-injection of rflaA:Ova resulted in a 2-fold higher IgG2a production one week after the first immunization (Figure 63 A). However, one week after the second sensitization or after the Ova-pellet challenge no differences between both

application routes were detectable (Figure 63 B and C). For all investigated time points Ova-specific IgG2a levels were approximately two fold lower for non-vaccinated or Ova-vaccinated animals compared to rflaA:Ova-vaccinated groups (Figure 63).

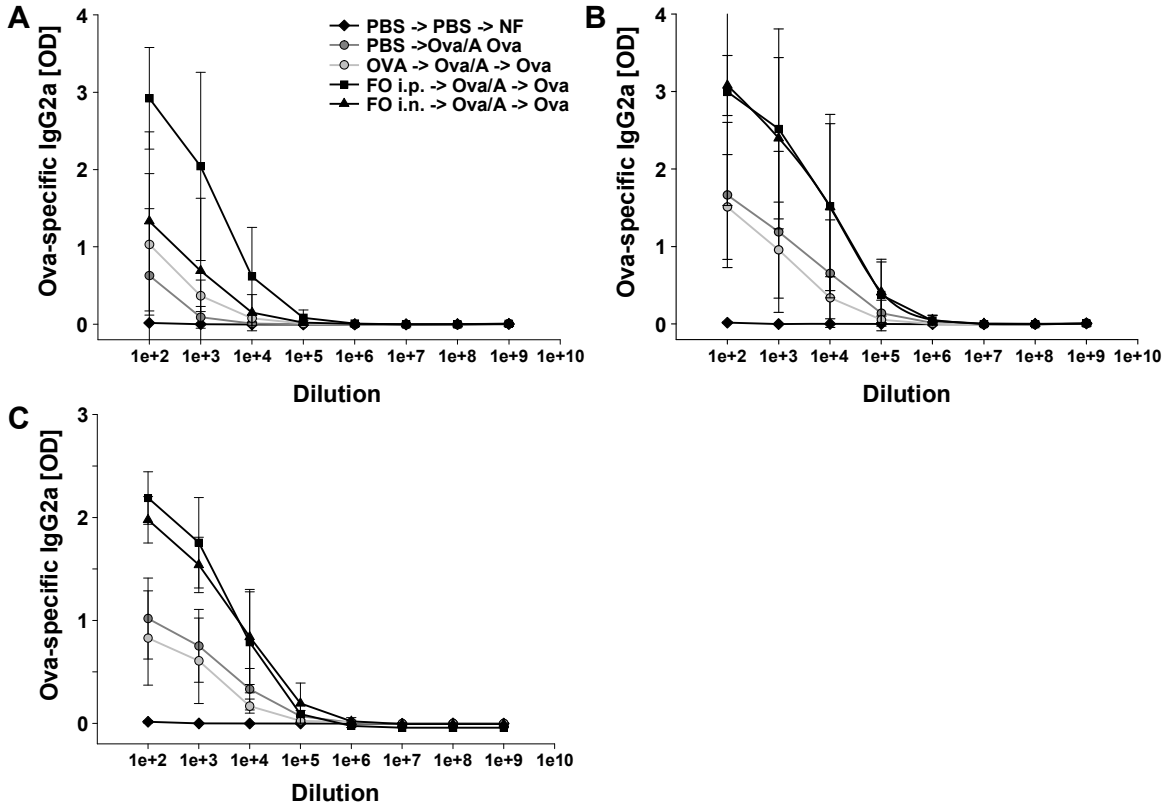


Figure 64: Intranasal administration of rflaA:Ova induces Ova-specific IgG2a production. Control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were continuously challenged with either Ova-containing pellets (Ova) or normal pellets (NF) for 8 days. Levels of Ova-specific IgG2a antibodies in the sera were determined one week after the first sensitization (A), one week after the second sensitization (B), and at the end of Ova-pellet challenge (C) by ELISA (n = 6 mice per group). Group nomenclature: (vaccination -> sensitization -> challenge).

When investigating Ova-specific IgE levels the suppression of IgE-production upon prophylactic vaccination with rflaA:Ova described under (4.3.2.7) could be reproduced (Figure 65). Here a steady increase over time in Ova-specific IgE levels was observed in non-treated or Ova-vaccinated animals, whereas Ova-specific IgE production was first detected after Ova-pellet challenge in rflaA:Ova-vaccinated animals (either i.n or i.p., Figure 65). Compared to i.p.-injection the potency of rflaA:Ova to suppress IgE-production was slightly increased upon intranasal administration (OD 0.9 for i.n. vs 1.6 for i.p., Figure 63 C). At the end of Ova-pellet challenge IgE levels in rflaA:Ova-vaccinated animals were 1.75-(i.p.) to 3.1-fold (i.n.) lower than for either non-treated or Ova-vaccinated animals (Figure 65 C).

In summary, these results show that intranasal application of rflaA:Ova was also sufficient to prevent the establishment of intestinal allergy in the tested model. Here, intranasal application was shown to be even more efficient in suppressing Ova-specific IgE production than i.p.-injection.

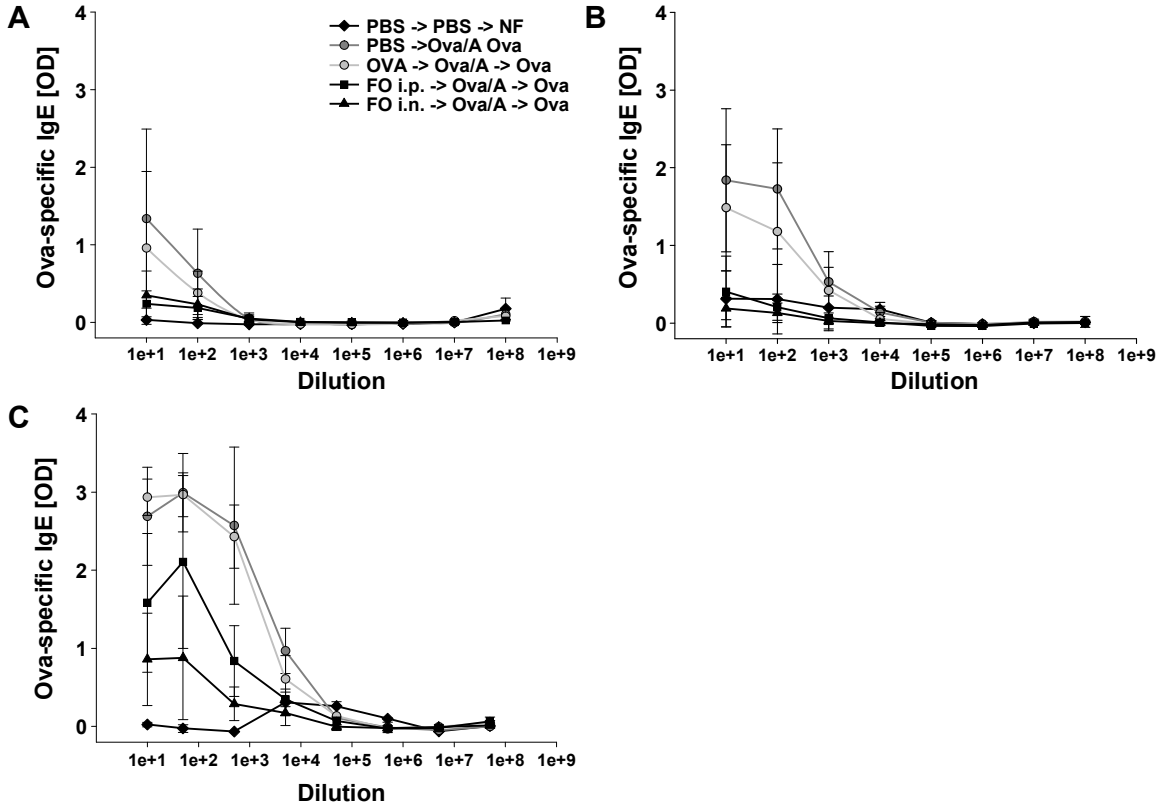


Figure 65: Intranasal administration of rflaA:Ova suppresses Ova-specific IgE production. Control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were continuously challenged with either Ova-containing pellets (Ova) or normal pellets (NF) for 8 days. Levels of Ova-specific IgE antibodies in the sera were determined one week after the first sensitization (A), one week after the second sensitization (B), and at the end of Ova-pellet challenge (C) by ELISA (n = 6 mice per group). Group nomenclature: (vaccination -> sensitization -> challenge).

4.3.3 Evaluation of rflaA and rflaA:Ova in a therapeutic in vivo model of Ova-induced intestinal allergy

In a preliminary experiment the therapeutic potential of the different constructs for the treatment of an established allergic response was investigated using the model of severe Ova-induced intestinal allergy. For this purpose mice were first sensitized to Ova adsorbed to aluminium hydroxide (twice by i.p.-injection), then treated twice by i.p.-injection with equimolar anmounts of Ova (10 µg), rflaA (6.9 µg), rflaA plus rOva (6.9 µg + 10 µg), or rflaA:Ova (16.9 µg), and finally challenged for 5.5 to 6.5 days with Ova-containing food

pellets. During Ova-pellet challenge mice were monitored for signs of illness, weight loss, core body temperature, and food uptake on a daily base.

For therapeutic vaccination treatment groups were abbreviated by including the construct used for sensitization, therapeutic vaccination, and the challenge in chronological order (see 3.3.3). Meaning that in the Ova/A -> rflaA:Ova -> Ova group animals were first sensitized to Ova with alum, then vaccinated with rflaA:Ova, and finally challenged with Ova-containing food-pellets.

4.3.3.1 Therapeutic vaccination with rflaA:Ova slightly reduces disease symptoms

In accordance with the results presented for the prophylactic vaccination the allergic positive control group (Ova/A -> PBS -> Ova) displayed the same signs of illness, namely ruffed fur, reduced mobility, and increasing softness of faeces during the time of Ova-pellet challenge. No comparable signs of illness were observed for mice receiving normal food (PBS -> PBS -> NF, Ova/A -> Ova -> NF, data not shown).

When comparing mouse phenotypes between the different treatment groups all groups showed comparable signs of illness. However, softness of faeces was less pronounced in rflaA:Ova-vaccinated animals compared to the other groups. In contrast to the results obtained for the prophylactic vaccination studies rflaA:Ova-vaccinated mice also showed ruffed fur and reduced mobility (data not shown). In accordance with these observations rflaA:Ova-vaccinated animals displayed a significantly reduced symptom score on day 5.5 of Ova-pellet challenge (Figure 66). Here, a constant increase of symptom scores over the time of Ova-pellet challenge was observed for either non-vaccinated (Ova/A -> PBS -> Ova), Ova- (Ova/A -> Ova -> Ova), rflaA- (Ova/A -> rflaA -> Ova,), or rflaA plus Ova-vaccinated groups (Ova/A -> rflaA + Ova -> Ova, Figure 66). No symptoms were detected in non-vaccinated and non-challenged controls (PBS -> PBS -> NF, Figure 66).

In summary, therapeutic vaccination with rflaA:Ova was shown to result in slightly reduced symptom scores due to less pronounced softness of faeces, although rflaA:Ova-treated animals showed phenotypic signs of illness.

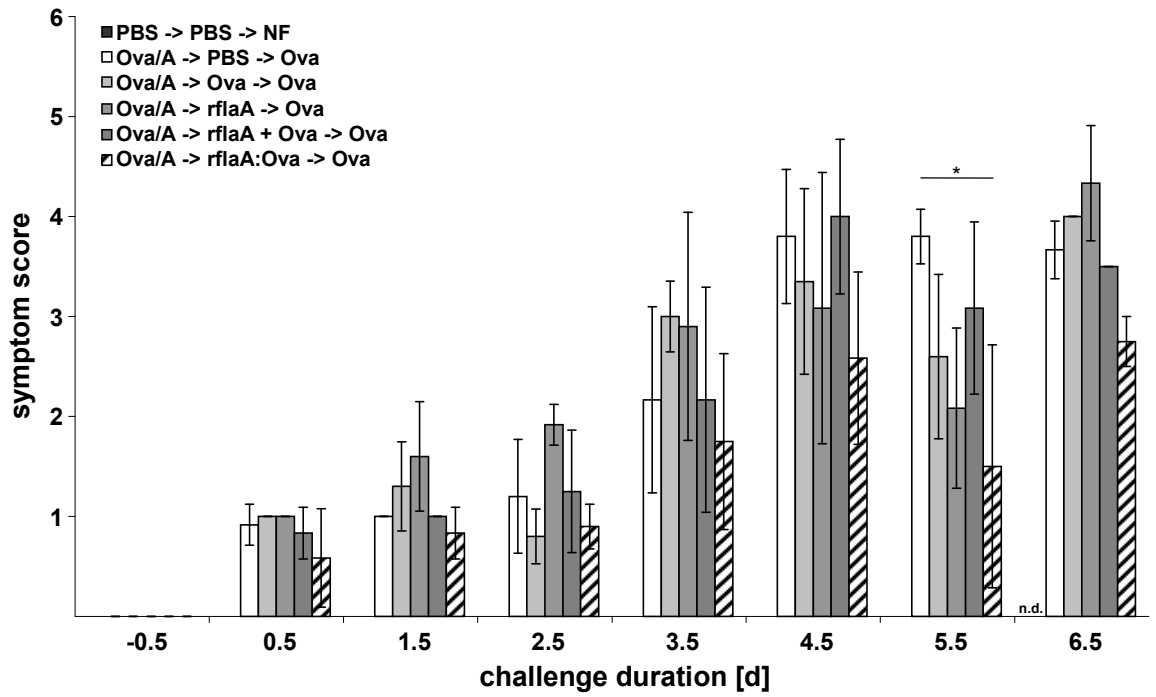


Figure 66: rflaA:Ova vaccinated mice show a slightly reduced symptom score. Control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were continuously challenged with either Ova-containing pellets (Ova) or normal pellets (NF) for 6.5 days. Disease symptoms were evaluated for every mouse on a daily base. Mean symptom scores were calculated for each group (n = 6 mice per group). n.d. not determined. Group nomenclature: (sensitization -> vaccination -> challenge).

4.3.3.2 rflaA:Ova-vaccinated mice show a higher food uptake

Next the influence of Ova-pellet challenge on food uptake was investigated (Figure 67). Before challenge all groups displayed a similar mean pellet uptake of about 2 to 2.5 g per day and mouse (Figure 67). In non-vaccinated, Ova-, rflaA-, and rflaA plus Ova-vaccinated animals mean Ova-pellet uptake was reduced to 1 to 1.7 g between days 3.5 and 5.5 of challenge. In contrast to this, in rflaA:Ova-vaccinated mice daily food uptake constantly ranged between 2.25 and 2.75 g per mouse (Figure 67). For non-challenged control mice (PBS -> PBS -> NF) a similar food uptake between 2.5 and 3.5 g per mouse and day (Figure 67) was recorded. Overall food uptake rates were comparable to the results obtained from prophylactic vaccination experiments (see Figure 51). Overall uptake of Ova protein was calculated to range from 0.3 (on days 3.5 to 5.5) to 0.8 g (on day 0.5) of Ova per (mouse and) day.

Taken together these results show that in accordance with the slightly reduced symptom scores in rflaA:Ova-treated animals food uptake rates were comparable to unchallenged animals, whereas the other treatment groups displayed a reduced food uptake.

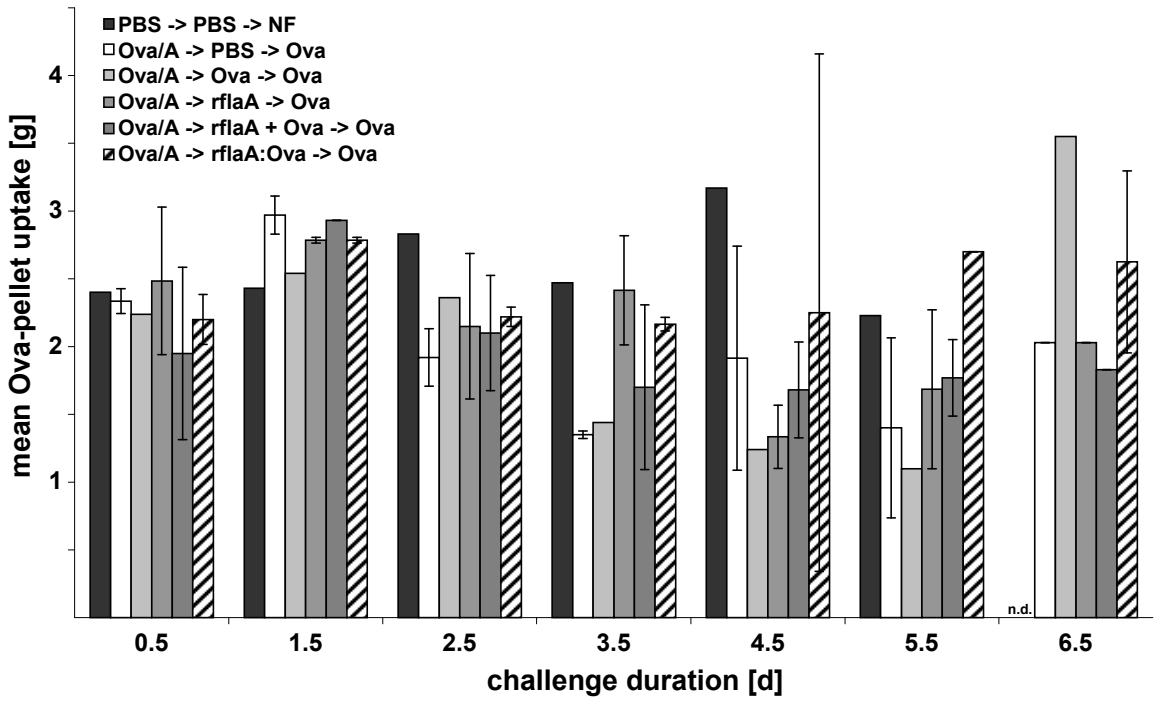


Figure 67: rflaA:Ova vaccinated mice show a constant food uptake. Control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were continuously challenged with either Ova-containing pellets (Ova) or normal pellets (NF) for 6.5 days. Food pellets were weighted every day and mean pellet uptake per mouse and day was calculated for each group (n = 6 mice per group). n.d. not determined. Group nomenclature: (sensitization -> vaccination -> challenge).

4.3.3.3 Therapeutic application of rflaA:Ova does not prevent weight loss or temperature drop

In contrast to the results obtained for the prophylactic vaccination study (see Figure 52) in this experimental setting therapeutic vaccination with the fusion protein was not sufficient to prevent weight loss induced upon Ova-pellet challenge. Hereby, all challenged groups displayed similar weight loss rates (Figure 68).

Moreover, therapeutic vaccination with none of the used constructs was sufficient to prevent the drop of core body temperature induced by Ova-pellet challenge in sensitized mice (Figure 69). Hereby, in all challenged groups a temperature drop of approximately 3°C was observed on day 5.5, whereas the non-challenged control group (PBS -> PBS -> NF) showed no change in core body temperature (Figure 69).

In conclusion in the used vaccination scheme none of the tested constructs was able to prevent weight loss and temperature drop induced upon Ova-pellet challenge.

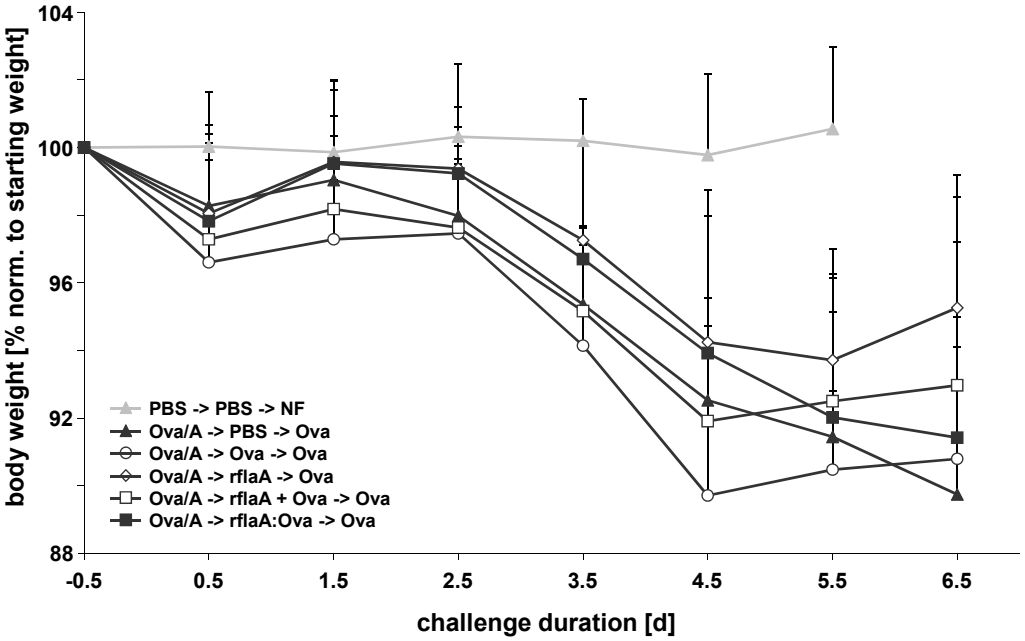


Figure 68: All therapeutic treatment groups display a similar weight loss. Control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were continuously challenged with either Ova-containing pellets (Ova) or normal pellets (NF) for 6.5 days. Body weight for every mouse was determined, normalized to the individual starting weight, and mean normalized body weights were calculated for each day and group (n = 6 mice per group). Group nomenclature: (sensitization -> vaccination -> challenge).

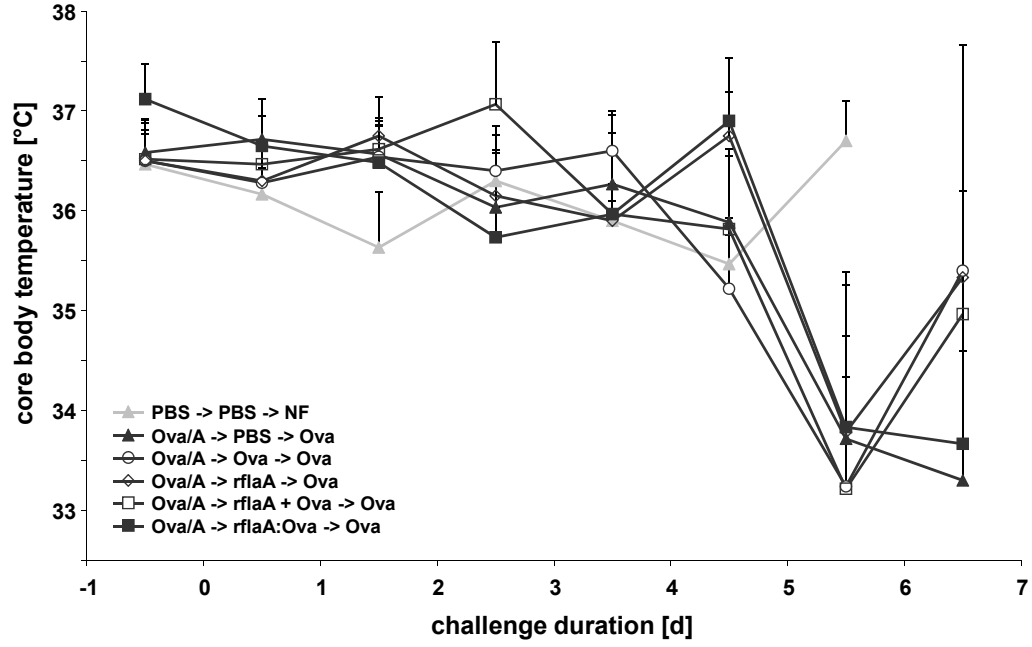


Figure 69: rflaA:Ova vaccination does not protect against temperature drop. Control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were continuously challenged with either Ova-containing pellets (Ova) or normal pellets (NF) for 6.5 days. Core body temperatures for every mouse were determined rectally and mean body temperatures were calculated for each day and group (n = 6 mice per group). Group nomenclature: (sensitization -> vaccination -> challenge).

4.3.3.4 Therapeutic vaccination with rflaA:Ova does not induce differences in T cell proliferation

To investigate the influence of therapeutic vaccination on T cell priming and responses upon restimulation, CD4 T cells were isolated from spleens and mesenteric lymph nodes (MLN) from vaccinated and control groups (see 3.3.4). The purified T cells were co-cultured with syngenic APC and restimulated with Ova (Repository figure 3 and Repository figure 4). In accordance with the results obtained in the prophylactic vaccination study due to unknown reasons unstimulated co-cultures of both splenic and mesenterial lymph node CD4 T cells displayed rather high proliferation rates (Repository figure 3 and Repository figure 4). Therefore, when comparing T cell proliferation between the different vaccination groups no differences in Ova-induced proliferation were detectable (Repository figure 3 and Repository figure 4). Moreover, on day 5.5 of Ova-pellet challenge numbers of MLN cells were not sufficient to perform the proliferation assay.

4.3.3.5 Therapeutic vaccination does not influence T_{reg} frequency

When investigating frequencies of regulatory T cells in spleens either on day 5.5 and 6.5 of Ova-pellet challenge no pronounced differences in T_{reg} frequencies were detected between the different treatment groups (Figure 70).

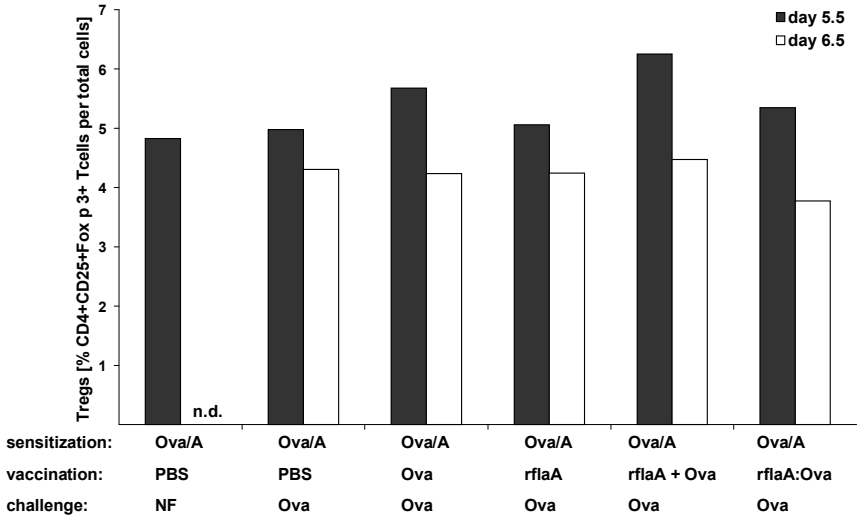


Figure 70: rflaA:Ova-vaccination does not alter T_{reg} frequency. On day 5.5 (black) and 6.5 (white) of Ova-pellet challenge spleens from 3 animals per group were dissected under sterile conditions, ground, filtered, and pooled. After lysis of red blood cells 10^6 cells were stained for $CD4^+CD25^+$Fox p 3^+ T_{regs} using the T_{reg} staining kit. Cells were gated on CD4 T cells and the frequency of $CD4^+CD25^+$Fox p 3^+ T_{regs} was determined by analyzing 10^5 T cells. N.d. not determined.

In MLN isolates overall T_{reg} frequencies were higher than in corresponding spleen samples, ranging from 5 to 7.5%. On day 5.5 the highest percentages of Fox p 3 positive regulatory T cells were observed in non-challenged (Ova/A -> PBS -> NF, 7.5% T_{reg} frequency) and non-vaccinated (Ova/A -> PBS -> Ova, 7% T_{reg} frequency) control groups, whereas in direct comparison Ova- (Ova/A -> Ova -> Ova, 6.2%), rflaA- (Ova/A -> rflaA -> Ova, 6%), rflaA plus Ova- (Ova/A -> rflaA + Ova -> Ova, 5.2%), and rflaA:Ova-vaccinated groups (Ova/A -> rflaA:Ova -> Ova, 5.9%) showed slightly reduced T_{reg} frequencies (Figure 71). Hereby, T_{reg} frequencies were lowest for mice receiving the mixture of rflaA and Ova (5.2% T_{reg} frequency). Therefore, in contrast to splenic samples vaccination slightly reduced T_{reg} numbers compared to control groups. In accordance with the results obtained for splenic isolates on day 6.5 of Ova-pellet challenge all investigated treatment groups showed equal T_{reg} frequencies in mesenteric lymph nodes (Figure 71).

Whereas no differences in T_{reg} frequencies could be detected in spleen samples, in MLN vaccinated groups (either Ova-, rflaA-, rflaA + Ova-, or rflaA:Ova-treated) displayed reduced T_{reg} numbers on day 5.5 but not 6.5 of Ova-pellet challenge.

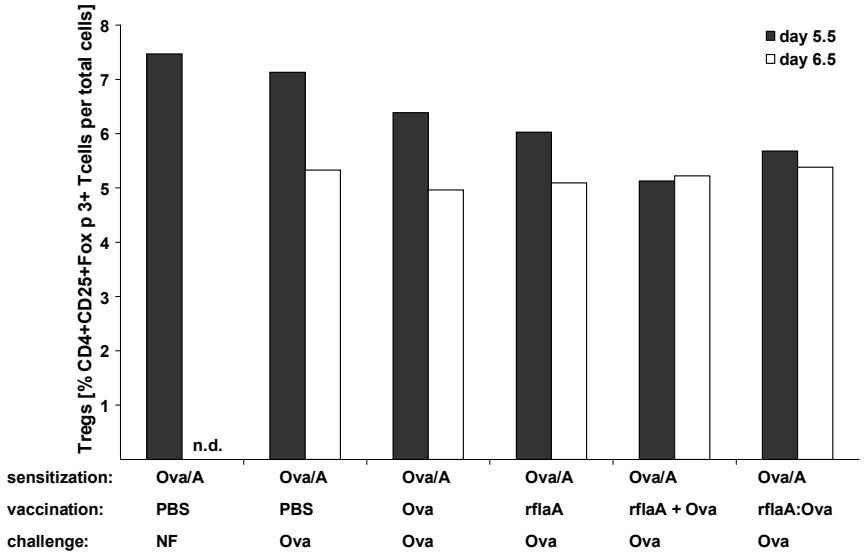


Figure 71: Vaccination reduces T_{reg} frequency on day 5.5. On day 5.5 (black) and 6.5 (white) of Ova-pellet challenge MLN of 3 animals per group were dissected under sterile conditions, ground, filtered, and pooled. After lysis of red blood cells 10^6 cells were stained for $CD4^+CD25^+$Fox p 3^+ T_{regs} using the T_{reg} staining kit. Cells were gated on CD4 T cells and the frequency of $CD4^+CD25^+$Fox p 3^+ T_{regs} was determined by analyzing 10^5 T cells.

4.3.3.6 Therapeutic vaccination with rflaA:Ova reduces T cell activation

On day 5.5 of Ova-pellet challenge CD4 and CD8 T cells showed highest CD62-L expression in non-challenged mice followed by rflaA:Ova-vaccinated animals, whereas for the other treatment groups no differences in expression levels to non-vaccinated control animals (Ova/A -> PBS -> Ova) were detected (Figure 72 A). On splenic CD4 T cells a reduction of PD-1 expression in rflaA- and rflaA plus Ova-vaccinated animals was detected, which was further reduced in rflaA:Ova treated animals, resulting in comparable PD-1 expression to non-challenged control animals (Ova/A -> PBS -> NF, Figure 72 A). Ova-treated animals showed no differences in PD-1 expression compared to non-vaccinated control animals. For CD11c dendritic cells no striking differences in surface marker expression were detected (Repository figure 13), whereas for CD19 B cells a slight down-regulation of CD86 and MHC II expression in non-challenged (Ova/A -> PBS -> NF) compared to non-vaccinated mice was detected (Repository figure 13). Additionally, MHC II expression was slightly down-regulated in B cells derived from rflaA plus Ova treated mice.

On day 6.5 all vaccination groups demonstrated increased levels of CD62-L expression on both splenic CD4 and CD8 T cells compared to non-vaccinated animals (Ova/A -> PBS -> Ova, Figure 72 B). Here, CD62-L expression was highest for rflaA:Ova-vaccinated animals, slightly lower for rflaA plus Ova-vaccinated animals, and again lower for Ova- and rflaA-vaccinated groups (Figure 72 B). No differences compared to the non-vaccinated (Ova/A -> PBS -> Ova) group were detected for the investigated markers on either B cells or dendritic cells (Repository figure 14).

On day 5.5 of Ova-pellet challenge all vaccinated groups displayed a slight reduction of PD-1 expression on splenic CD4 T cells (Figure 73 A). In accordance with the results obtained from MLN on day 6.5 the highest levels of CD62-L expression were observed in rflaA:Ova-vaccinated animals on both investigated T cell subsets (Figure 73 A).

On CD19 B cells higher expression levels of the activation markers CD69, CD80, and CD86 were observed in non-challenged animals (Ova/A -> PBS -> NF) compared to non-vaccinated but challenged animals (Ova/A -> PBS -> Ova, Repository Figure 15). In contrast to this, CD40 and MHC I were slightly down-regulated in non-challenged compared to non-vaccinated animals. For Ova-, rflaA-, rflaA plus Ova-, and rflaA:Ova-treated groups no differences in expression levels were detected for the investigated surface markers (Repository figure 15). Dendritic cells of rflaA-, rflaA plus Ova-, and rflaA:Ova-vaccinated animals displayed a reduced MHC II expression compared to non-vaccinated animals (Ova/A -> PBS -> Ova), whereas non-challenged animals had even higher MHC II levels (Repository figure 15).

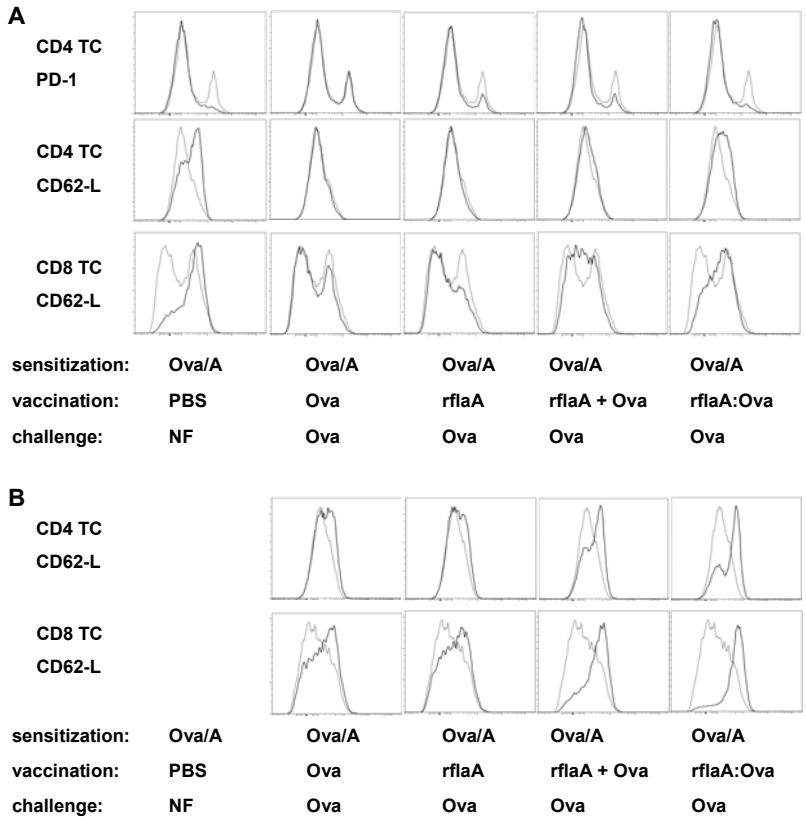


Figure 72: Therapeutic rflaA:Ova vaccination reduces splenic TC activation on day 5.5. On day 5.5 (A) or 6.5 (B) of Ova-pellet challenge animals were sacrificed, spleens were prepared, and three animals per group were pooled. Subsequently, red blood cells were lysed and expression levels of the indicated lineage and cell surface markers were determined by flow cytometry and compared to the PBS -> Ova (allergic positive control, grey) group.

In accordance with the results obtained from splenic cells on day 6.5 lymph node derived T cells displayed highest levels of CD62-L expression in rflaA:Ova-vaccinated animal followed by rflaA plus Ova, Ova, and rflaA treated groups (Figure 73 B). Hereby, no differences between CD4 and CD8 T cell subsets were detected. No differences in marker expression were detected on either B cells or dendritic cells (Repository figure 16).

Therefore, in accordance with the results presentend in (4.3.2.5) for the prophylactic vaccination rflaA:Ova-treated animals displayed a reduced CD4 and CD8 T cell activation as determined by high levels od CD62-L expression. For B cells and DC no pronounced differences in surface marker expression could be detected.

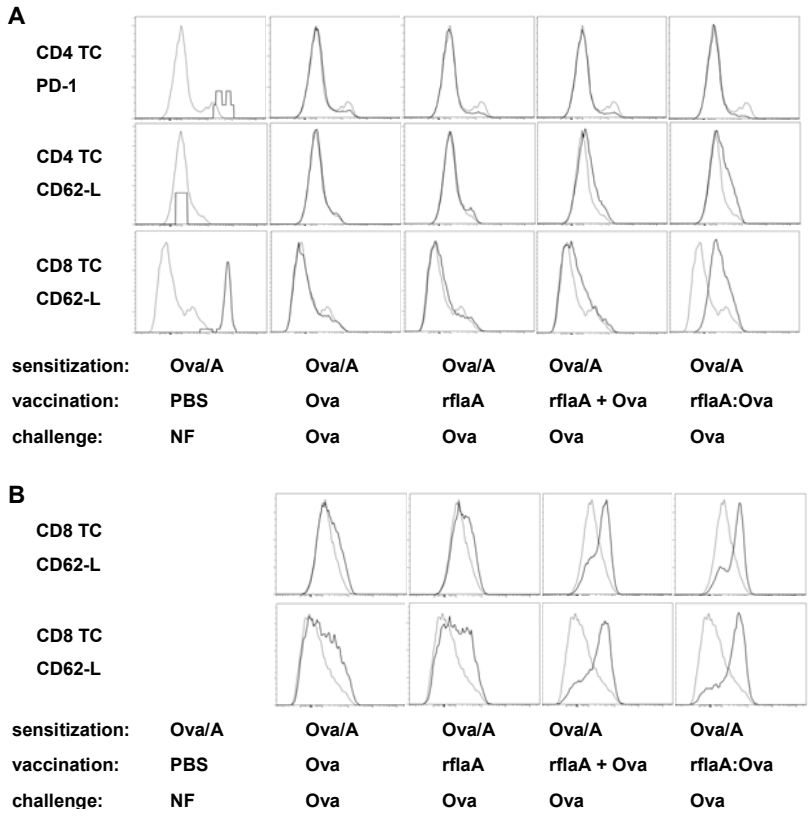


Figure 73: Therapeutic application of rflaA:Ova reduces TC activation in MLN. On day 5.5 (A) and 6.5 (B) of Ova-pellet challenge animals were sacrificed, mesenteric lymph nodes were prepared, and three animals per group were pooled. Subsequently, red blood cells were lysed and expression levels of the indicated lineage and cell surface markers were determined by flow cytometry and compared to the PBS -> Ova (allergic positive control) group.

4.3.3.7 Therapeutic vaccination is not sufficient to alter established Ova-specific antibody responses

Upon therapeutic vaccination with the different constructs no differences in Ova-specific IgG1-levels were detectable (Repository figure 9). Additionally, analysis of Ova-specific IgG2a or IgE levels revealed no differences between the different therapeutic vaccination groups (Repository figure 10 and Repository figure 11). Accordingly, no differences in Ova-specific IgG2a/IgE ratios were detected. Here all therapeutic treatment groups displayed lower IgG2a/IgE ratios than the untreated control group (PBS -> PBS -> NF, Repository figure 12) indicating the induction of Ova-specific Th2-biased immune responses.

5. Discussion

5.1 Flagellin-containing fusion proteins are potent immune modulators

In this study flagellin A, a TLR5-ligand derived from *Listeria monocytogenes,* was tested for its capacity to prevent and treat allergy. Flagellin was selected as it serves both as an adjuvant and a carrier molecule (180,181). Flagellins are characterized by their ability (i) to activate TLR5-expressing cells which may lead to an enhanced immune response to foreign epitopes, (ii) to improve immune responses by local mucosal delivery, and (iii) to express multiple copies of foreign proteins or epitopes which is unique to the polymeric nature of the bacterial flagellum (156,181). Consequently, TLR5-ligand flagellin was hypothesized to serve as a prophylactic and/or therapeutic adjuvant for the treatment of type I allergies. The strategy was to further increase the adjuvant effect of flagellin by conjugation of flagellin to an allergen of choice. Here, the proximity of the TLR-ligand to an antigen could promote the adjuvant effect by simultaneously targeting and activating the same effector cell. Flagellin is the only relevant proteinous TLR-ligand known, which can be fused to any antigen of interest using recombinant DNA-techniques.

Listeria monocytogenes flaA was chosen as model flagellin because heat killed *Listeria* preparations were demonstrated to activate innate immunity in a TLR-dependent way, resulting in the modulation of established allergic responses (see 1.4.2.1 and 1.4.2.2). Moreover, flaA does not contain the central hypervariable part present in most flagellin molecules (for example *Salmonella* fliC). The hypervariable region does not contribute to TLR5-activation, but is known to be an antigenic determinant for antibody induction (141,147,150). Anti-flagellin antibodies might neutralize the adjuvant effect of flagellin based vaccines and are therefore considered detrimental. Consequently, *Listeria monocytogenes* flaA can be considered a “naturally optimized” flagellin for vaccine development. Ovalbumin was chosen as model allergen since it is both, a well established model antigen for immunological studies and a food allergen (Gal d 2). Here, the availability of Ova specific tools, such as mice expressing an Ova-specific T cell receptor (DO11.10, OT-II) allow more detailed analysis of the fusion proteins’ mode of action. Moreover, egg allergy is a common food allergy in childhood found in approximately 2% of children and 1% of adults (157). All proteins were produced recombinantly in *E. coli*, and purified with negligible endotoxin contaminations and considerable amounts of secondary structure elements as determined by CD-spectroscopy. Additionally, all flagellin-containing constructs were shown to be able to activate both human and murine TLR5 using TLR5 expressing HEK293 reporter cells.

5.2 rflaA:Ova has potent immune modulating properties *in vitro*

5.2.1 rflaA:Ova suppresses Th1 and Th2 cytokine secretion in vitro while preserving IL-2 production

The fusion protein was found to efficiently repress both Th1-type cytokine IFN-γ (in DO11.10 and OT-II derived cells) and Th2-type cytokines IL-4 and IL-5 (only in DO11.10 cells, see 4.2.9 and 4.2.12) upon stimulation of Ova-specific CD4 T cells co-cultured with mDC. Interestingly, this suppression of Th1 and Th2 cytokines was not observed when mDC and CD4 T cells were stimulated with a mixture of rflaA plus rOva or rflaA alone. This is of particular interest, since it is unlikely that the conjugation of rflaA to rOva reduced T cell immunogenicity of the allergen, as the fusion protein induced higher IL-2 production from Ova-specific CD4 T cells compared to rOva alone. Consistent with these data (see 4.2.9) Bates et al. reported adoptively transferred OT-II cells stimulated with flagellin:Ova to show a 4- to 5-fold enhanced IL-2 production compared to cells stimulated with Ova alone (182). However, previous contradictive reports using flagellin as an adjuvant showed either inhibition of Th2 responses without inducing a Th1 response (140) or induction of polarized Th2 responses by suppressing Th1 responses (183).

5.2.2 rflaA:Ova strongly induces IL-10 secretion from mDC

Of note, it was shown by ELISA and intracellular cytokine staining that rflaA:Ova stimulation induced a strong IL-10 production by mDC which was not observed upon administration of either rflaA alone or the mixture of both components (see 4.2.2 and 4.2.3). IL-10 is an anti-inflammatory cytokine reported to suppress both Th1 and Th2 cytokine production by CD4 T cells (184,185). Thus, IL-10 produced by rflaA:Ova-stimulated mDC is likely to account for the observed inhibition of Th1 and Th2 cytokine production by Ova-specific CD4 T cells. In line with this finding, low concentrations of rflaA:Ova were shown to induce IFN-γ secretion from OT-II CD4 T cells, whereas higher rflaA:Ova concentrations resulted in IL-10 production by mDC. IL-10 dose-dependently suppressed Ova-induced IFN-γ secretion (see 4.2.12). Additionally, recombinant IL-10 exogenously added to mDC:CD4 T cell co-cultures was able to reduce both T cell-derived IL-4 and IFN-γ production (see 4.2.11). Finally, neutralization of rflaA:Ova-induced IL-10 production by mDC using neutralizing antibodies dose-dependently restored IFN-γ secretion. Therefore, fusion protein induced IL-10 secretion was shown to influence the differentiation of naïve antigen-specific T cells (see 4.2.11).

To investigate whether the fusion protein was able to suppress cytokine secretion from biased T cells as from naïve antigen-specific T cells (see 4.2.14), BALB/c mice were immunized with Ova adsorbed to aluminium hydroxide in order to induce Th2-biased Ova-specific T cell differentiation (see 4.2.14). Isolated splenic CD4 T cells co-cultured with syngenic mDC produced considerable amounts of Th2 cytokines (IL-4, IL-5) when stimulated with Ova, indicating the successful generation of Ova-specific Th2-biased T cells. Interestingly, stimulation with the fusion protein did not induce comparable cytokine secretion. Moreover, when co-cultures were stimulated with Ova and the fusion protein at the same time rflaA:Ova was able to suppress the Ova-induced secretion of both Th2 and Th1 cytokines. These results clearly show the suppression of cytokine secretion from Th2-biased T cells mediated by the fusion protein. This is of particular importance as it reflects the pathological situation in allergic patients. Here, fusion protein-induced IL-10 production might be an effective strategy to suppress allergen-specific reactions in patients. However, extensive safety studies need to be performed in order to exclude exaggerated mast cell activation by aggregated flagellin:allergen fusion proteins.

Suppression of allergen-specific Th2 responses is one strategy for specific immunotherapy of type I allergies. Over the last years there has been cumulating experimental evidence that in addition to T cell derived IL-10, other cell types, such as DC, NK cells, or B cells are also capable of producing the immunosuppressive cytokine IL-10 (186-189). Similar to the results presented in this study, Ureta and coworkers showed BMDC differentiated in the presence of vitamin D3 to secrete IL-10 and induce a subpopulation of $CD4^+CD25^+CD62\text{-}L^+$Fox p 3^+ regulatory T cells (190). Moreover, Thilo Jacob and co-workers showed that suppression of allergic responses mediated by IL-10 were still observed when either B cells, T cells, or both were deficient for IL-10 production (unpublished, presented on the 4^{th} ISMA meeting 2010 in Munich), suggesting that other cell types such as DC or macrophages are able to produce significant amounts of IL-10. These data support our findings of mDC-derived IL-10 contributing to the suppression of allergic responses.

The goal of specific immunotherapy for allergies is to either re-establish immunological tolerance or to induce an immune deviation towards more Th1-dominated immune responses. However, recent findings have shown that a strong and predominant induction of allergen-specific Th1 responses in immunotherapy of allergic diseases might rather promote unwanted allergic inflammation *in vivo* (192). In contrast to these findings, during this study no adverse effects were observed when administrating rflaA:Ova *in vivo*. Therefore, recombinant flaA:allergen fusion proteins which can suppress both Th1 and Th2 cytokine production from CD4 T cells, might be promising and safe vaccines candidates to improve the intervention of type I allergies.

5.2.3 Flagellin fusion proteins do not induce Th17 biased immune responses

Interestingly, high levels of IL-6 production, confirmed by intracellular cytokine staining (see 4.2.2 and 4.2.3) were observed upon stimulation of mDC with rflaA:Ova, rflaA:Pru p 3, and to a lesser extend rflaA:Ara h 2. Of note, in addition to IL-23 (13,14), the pro-inflammatory cytokine IL-6 is critically involved in Th17 cell differentiation (15). It has been shown that co-culture of $CD11b^{hi}CD11^{hi}$ lamina propria DC and CD4 T cells induced functional Th17 cell differentiation upon stimulation with flagellin (193). Recent studies suggested extensive activation of Th17 cells to be the cause for some autoimmune diseases (16,17). Therefore, the induction of Th17-biased T cells by the fusion proteins might have detrimental effects *in vivo* and should be critically evaluated. However, no TGF-β secretion and only minor IL-23 production were detected upon administration of the different proteins. Consequently, upon co-culture of mDC with Ova-specific CD4 T cells rflaA:Ova stimulation did not result in IL-17A production different from the one induced by the equimolar amount of ovalbumin alone (see 4.2.10). Moreover, the application of the different constructs *in vivo* did not result in detectable IL-17 secretion at any of the investigated time points (data not shown). Therefore, the administration of the fusion constructs used in this study does not result in Th17 biased immune responses.

5.2.4 Flagellin fusion proteins strongly activate murine mDC

In addition to remodeling CD4 T cell cytokine responses, direct effects of rflaA fusion proteins on the activation and maturation status of stimulated mDC were observed. The different fusion proteins (rflaA:Ova, rflaA:Pru p 3, and rflaA:Ara h 2) triggered up-regulation of CD40, CD69, CD80, and CD86, whereas rflaA alone or the equimolar mixture of rflaA with recombinant allergens did not show comparable effects (see 4.2.2 and 4.2.16).

Although rflaA:Ara h 2 and rflaA:Pru p 3 fusion proteins strongly activated mDC their potency to induce cytokine (especially IL-10) secretion was limited compared to rflaA:Ova (see 4.2.16). Here, it is hypothesized that high molecular aggregates might be superior in inducing IL-10 secretion compared to non-aggregated proteins (see discussion of PLP1 in 5.2.8). In line with this, reduction and alkylation restored TLR5-activation capacities of rflaA:Ara h 2 and rflaA:Pru p 3 to levels observed for equimolar amounts of rflaA alone, whereas for the higher molecular weight fusion protein rflaA:Ova partial defolding of Ova could not enhance TLR5-binding (see 4.1.7) In order to further clarify these findings additional experiments will be necessary to quantify size and degree of high molecular aggregates in flagellin fusion protein preparations (see 7).

To further characterize the potency of rflaA:Ova to activate murine mDC rflaA:Ova was compared to other TLR-ligands (see 4.2.8). Here, protein amounts as low as 2.5 µg rflaA:Ova were shown to induce mDC activation and cytokine secretion (IL-1ß, IL-6, and IL-10) comparable to strong DC activators such as e.g. LPS and resiqimod (R848, see 4.2.8). Compared to a heat killed *Listeria* preparation rflaA:Ova induced IL-1ß secretion comparable to 10^7 HKLM, IL-6 secretion comparable to 10^7 to 10^8 HKLM, and a lower but still remarkable IL-10 production. Interestingly, no mDC activation was observed upon stimulation either with Pam_3CysK_4, HKLM, or FSL-1 although these TLR-ligands induced considerable cytokine secretion (Figure 30). All these TLR-ligands induced rather strong cytokine secretion, therefore it may be hypothesized that after 24 h of stimulation mDC became apoptotic due to this strong activation, a protective mechanism to prevent extensive inflammation. Therefore, the rflaA:Ova fusion protein was shown to be a strong activator of mDC equivalent to well established stimuli as LPS and R848.
In contrast to this *L.m.* flagellin A was shown to be a rather weak stimulus for mDC. However, the ability of flagellin to activate DC is controversially discussed. Means and co-workers reported purified bacterial flagellin to be unable to mature murine splenic DC *in vitro* due very low TLR5 expression determined by qRT-PCR (194). On the other hand, Didierlaurent et al. (183) reported monomeric *Salmonella* flagellin C to induce up-regulation of CD40, CD80, and CD86 on splenic DC, suggesting DC-activation. In line with the strong cell activation and IL-10 secretion by rflaA:Ova stimulated mDC Edwards and co-workers demonstrated ligation of CD40 by TLR-stimulated DC to enhance IL-10 production (187).

Using TLR5-transgenic HEK293 cells rflaA showed a strong (TLR5-dependent) induction of IL-8 (see 4.1.7), comparable to the well-established TLR5-ligand *Salmonella typhimurium* rfliC. However, compared to equimolar amounts of the different fusion proteins its potency to activate DC maturation and cytokine secretion is either weak or not detectable in the concentrations applied (see 4.2.2). Here, flagellin A itself turned out to be only a weak stimulus. However, a significant IL-6 and IL-10 induction, mDC activation, and TLR5-up-regulation (see 4.2.2 and 4.2.5) was achieved using 10-fold higher rflaA concentrations, proving flagellins adjuvant capacities.

During the last years different flagellin fusion proteins were generated. In 2004 Cuadros and co-workers presented data on a flagellin:EGFP fusion protein. In line with the results presented in this thesis, this construct induced the maturation and secretion of pro-inflammatory cytokines (195). The flagellin:EGFP fusion protein was efficiently phagocytosed, processed, and presented by APCs and stimulated the proliferation of CD4 positive cells from EGFP-immunized mice *in vitro*, whereas antigens derived from rEGFP alone induced only minimal proliferation (195). In line with own results, these results show

that fusion to flagellin resulted in a strongly enhanced immunogenicity of the antigen and in immune responses directed against otherwise poorly immunogenic antigens. In a different study fusion of a deletion variant of the *Salmonella typhimurium* flagellin fliC (STFΔ2, lacking the hypervariable middle region) to the only poorly immunogenic ectodomain of the influenza matrix protein M2 (M2e) induced potent, long-lasting M2e-specific antibody responses which protected against otherwise lethal challenge with influenza A/Puerto Rico/8/34 (144). Of note, the antibody responses observed upon immunization with STFΔ2:M2e were both quantitatively and qualitatively superior to those observed upon administration of a mixture of both components, or M2e provided in alum (144).

While all these studies demonstrated a potent immune stimulatory capacity of flagellin fusion proteins using mouse *in vivo* models the underlying mode of action, for example the effect of fusion proteins on DC activation and maturation remained mostly unknown. In this thesis it was shown, that rflaA:Ova potently activated murine mDC *in vitro*, resulting in strong cell activation, TLR5-upregulation, and cytokine secretion. This strong cell activation is likely to explain the effects observed by others discussed above, therefore providing a potential mechanism for the superior immune stimulating effects of flagellin-containing fusion proteins.

In conclusion the results obtained in this thesis and in the studies mentioned above demonstrate a strongly increased immunogenicity of the fused antigens by the use of flagellin in fusion proteins.

5.2.5 The immune modulating effects are independent of LPS contaminations

Importantly, in this study any immunological effects of residual LPS can be excluded for all protein preparations used. Dose-dependent IL-6 and IL-10 secretion by mDC induced by LPS-administration revealed the residual amounts of LPS contained in the flaA:Ova preparation used (49.0 pg LPS in 16.9 µg rflaA:Ova) to have no impact on cell activation and cytokine secretion (see 4.2.4). In line with this, both, the protein concentration of rflaA and the mixture of rflaA with rOva used, which did not induce any of the investigated cytokines and only slight expression of activation markers, contained more overall LPS (75.9 pg LPS in 6.9 µg rflaA, 77.4 pg LPS in 6.9 µg rflaA + 10 µg rOva) than the amount of rflaA:Ova used. Finally, proteolytic digestion of rflaA:Ova using proteinase K, which degrades the protein while preserving LPS, abrogated IL-10 secretion from mDC (see 4.2.4), indicating this effect to be exclusively mediated by the protein and not by residual LPS contaminations. Therefore, the observed effects were clearly shown to be independent of LPS-contaminations.

5.2.6 Different flagellin fusion proteins display strong immunogenicity through TLR-mediated signaling

The performed experiments confirmed that the adjuvant activity of flaA:allergen fusion proteins triggers both the expression of co-stimulatory molecules as well as the secretion of different cytokines from mDC (see 4.2.2). In this way, the presentation of the antigen fused to flagellin is promoted to favor the induction of specific innate and adaptive immune responses. In line with these results, McDonald and co-workers stated that fusion of a West Nile Virus protein to flagellin was probably sufficient to fully activate antigen-specific B cells, resulting antibody-mediated protection against lethal viral challenge (144). The results obtained for the rflaA:Ova fusion protein are in agreement with other published studies using *Salmonella* flagellin C based fusion proteins as viral vaccines, showing that fusion of antigens to flagellin significantly increases immunogenicity and protective capacity of the fused antigen (142,145,196,197). Additionally, in accordance with the results presented in this thesis, in an independent study Bates et al. reported a (*Salmonella*) flagellinC:Ova fusion protein to have a superiour ability to induce IL-2 secretion, T cell clustering, and T cell proliferation compared to the equimolar mixture of both components. They explained their findings by a combination of antigen-targeting to TLR5 expressing DC and flagellin-induced signaling via TLR5 and MyD88 (182). In line with this hypothesis nanoparticles coated with flagellin were successfully used in an oral and subcutaneous vaccination strategy to target Ova to TLR5 positive APC and elicited systemic and mucosal immune responses (198). However, the exact mechanism by which the flagellin component exerts its adjuvant activity remained to be clarified.

The results obtained in this thesis provide additional information to explain these findings. Interestingly, mDC, but not pDC were found to express high levels of TLR5 upon stimulation with rflaA:Ova, whereas in equimolar concentrations flagellin alone or the equimolar mixture of both components did not induce TLR5 up-regulation (see 4.2.7). Moreover, recombinant flaA:Ova and rflaA did not induce production of cytokines and only slight up-regulation of co-stimulatory molecules in pDC (see 4.2.2) suggesting mDC to be the more important DC subset upon stimulation with flagellin containing fusion proteins. Using mDC derived from MyD88$^{-/-}$ mice the observed secretion of IL-6 and IL-10 was shown to be dependent on TLR-signaling (see 4.2.13). In co-culture experiments using rflaA:Ova stimulated MyD88$^{-/-}$ mDC in combination with Ova-specific OT-II CD4 T cells this lack of TLR-signaling (and rflaA:Ova-induced cytokine secretion) was shown to rescue the suppression of IFN-γ secretion observed in wild-type cells (see 4.2.13). Flagellin is known to be a natural agonist for at least three different innate immune receptors: TLR5 (128,199), Ipaf (ICE protease-activating factor) (200,201), and Naip5/Birc1e (neuronal apoptosis inhibitory protein) (202,203).

However, the observed immune modulating effects of flagellin fusion proteins are likely mediated mainly via TLR5 and DC since results obtained in this thesis using MyD88 deficient mice showed the immune modulating effects of rflaA:Ova to be dependent on TLR-signaling. Furthermore, Bates et al. have also shown the adjuvant effect of flagellin to be dependent on $TLR5^{+}CD11c^{+}$ cells *in vivo* (182). Taken together, these observations support both, TLR5 up-regulation and TLR5-mediated signaling, to be essential for rflaA:Ova-induced activation and maturation of mDC.

5.2.7 Co-application of TLR-ligands and antigen results in altered protein processing and presentation

In line with our results, Khan and co-workers showed that conjugation of Ova-peptides to Pam_3CysK_4 (a TLR2-ligand) or CpG-motifs (a TLR9-ligand) strongly enhanced antigen uptake, presentation, and DC-activation (204). Their results suggested the covalent linkage of peptide and TLR-ligands to be responsible for the enhanced uptake and activation in DCs. Moreover, using different inhibitors, the authors showed that the conjugates were translocated to the endosomal or lysosomal compartment independently of TLR-expression (204). Interestingly, in line with these observations Blander and Medzhitov (205) convincingly demonstrated that TLRs control the generation of antigen-derived T cell receptor ligands by influencing the phagocytotic processing of antigens delivered in the presence of TLR-ligands. In their hands, contents of phagosomes derived from microbial pathogens were preferentially presented by DC in the context of co-stimulatory signals, therefore providing a mechanism by which DC distinguish between self- and non-self derived antigens. Here, the recognition of non-self derived antigens relies on the simultaneous co-detection of danger signals such as PAMPs. These findings provide a potential explanation for the superior immune stimulatory properties of fusion proteins containing TLR-ligands, as presence of the TLR-ligand causes the preferential presentation of the fused antigen in the context of TLR-ligand induced co-stimulatory signals (205). In accordance with these observations, a *Salmonella* flagellinC:EGFP fusion protein was found to be efficiently internalized, processed, and presented by mouse APC (195). Consequently, BALB/c mice immunized with this flagellinC:EGFP construct developed specific CD4 and CD8 T cell responses against the otherwise non-immunogenic EGFP (195).

5.2.8 Aggregation of flagellin fusion proteins likely influences immunogenicity

In this thesis, high molecular aggregation products caused by intermolecular disulfide bonds were observed for all flagellin containing fusion constructs (see 4.1.5). These aggregations resulted in reduced TLR5 activation capacities (compared to an equimolar amount of rflaA alone, see 4.1.7) likely caused by sterical hinderance of flagellin binding to TLR5. This aggregation is probably favored by artificial flagella formation of the flagellin part during protein purification and refolding. Protein refolding starts under denaturating conditions. Here, with decreasing urea concentrations the flagellin part is likely to spontaneously form artificial flagella. During this self-assembly process unfolded allergen molecules (Ova, Pru p 3 or Ara h 2) are brought in close proximity to each other. This might result in arteficial intermolecular disulfide bonds between allergen molecules. FlaA itself does not contain cysteine residues and therefore does not form intermolecular disulphide bonds contributing to aggregation of the protein.

Protein aggregation likely influences antigen uptake, as well as subsequent processing and presentation by DC and therefore the fusion constructs immunogenicity. In line with the results presented in this thesis, Mizel and Bates reported the storage of flagellin fusion proteins to result in formation of large aggregates functioning in a TLR5 independent manner (206). Moreover, Cuadros et al. speculated that the flagellin:EGFP fusion protein does not only activate APC via TLR5 but also by increased internalization of the complex and subsequent differences in processing and presentation (195). Consistently, it was hypothesized that the size of (fusion) proteins influences processing by the APC, with larger proteins being slightly more effective regardless of the presence or absence of additional antigen (207). Concordantly, Zaborsky et al. could show that Bet v 1 dimerization induced at 37°C in the cell culture medium enhanced antigen uptake and DC activation (208). Legge et al. showed that strong aggregation observed for an Ig chimera carrying the encephalitogenic proteolipid protein (PLP) 1 induced IL-10 production by macrophages and DC (209). This resulted in the suppression of clinical symptoms in a mouse EAE model. The soluble, non-aggregated form of the chimeras did not induce IL-10 secretion (209). In line with these findings inhibition of endocytosis of rflaA:Ova by cytochalasin D and Bafilomycin A1 was shown to suppress rflaA:Ova-induced IL-6 secretion by mDC (see 4.2.15), suggesting that compared to the mixture of both components, the fusion protein is taken up more efficiently by DC.

Interestingly, in this thesis stimulation of DC with the different flagellin fusion proteins induced stronger mDC activation compared to flagellin alone. This may be explained by the fact that the aggregated fusion proteins contain a highly immunogenic TLR5-ligand and aggregation

of these fusion proteins leads to a very high concentration of flagellin molecules on a relatively small area of the DC surface. This high local concentration of antigen and DC activating TLR5-ligand is likely to result in both, the observed DC activation and stronger TLR-mediated protein uptake. Accordingly, besides being dependent on enhanced endocytosis the immune modulating effects of the fusion protein were shown to depend on TLR-signaling.

Ben-Yedida and Arnon reported isolated (non-covalently aggregated) flagella to be detectable in the blood up to 12 h post intramuscular administration whereas normally protein degradation in the body occurs within 30 minutes (157,209). These findings suggest flagellin to have a relatively long half live *in vivo* (157) and an increased stability compared to other proteins, possibliy due to the observed aggregation. This prolonged exposure in combination with the observed aggregation and TLR5-mediated pro-inflammatory signaling may result in enhanced activation of host immunity, which may in part explain the adjuvant effect of fusion proteins containing flagellin. However, bioavailability of the flaA fusion protein and pharmacokinetics was not addressed in the present study.

In summary, these observations suggest that, fusion of flagellin to proteinous antigens changes the resulting biophysical characteristics of the fusion proteins. This may include the stability or oligomerization state, which in turn may influence antigen uptake and processing in DC and therefore the kind of immune response induced. Additionally, covalent fusion of flagellin and antigen is likely to result in simultaneous uptake of both components by the same TLR5 positive target APC. This is not the case if both proteins are provided as a simple mixture. In the latter scenario, flagellin and Ova are likely taken up independently by different cells. Furthermore, even if a single cell internalizes both proteins the ratios of flagellin to Ova and therefore the type of immune response induced will be different from cell to cell. In the worst case this may result in bystander activation of Ova-specific Th2-responses by flagellin activated cells.

5.2.9 Partially defolded rflaA:Ova also is a potent immune modulator

In order to investigate the influence of both allergen folding and aggregation on the immune modulating properties of fusion proteins containing flagellin, in this study, a reduced and alkylated rflaA:Ova construct was generated, which displays a stable defolding of the ovalbumin part but is unlikely to alter the conformation of rflaA (see 4.1.6). This construct did not form any intermolecular disulfide bonds and therefore did not promote aggregation by covalent binding, but still displayed non-covalent flagellin mediated aggregation (detected by the proteins retention time upon size exclusion chromatography). Recombinant flaA:Ova

(R/A) demonstrated a slightly reduced hu/mTLR5 activation capacity when using low protein concentrations (see 4.1.7), but retained the capacity to activate mDC, induce cytokine secretion, up-regulate TLR5 on mDC, and stimulate CD4 T cells (see 4.2.2, 4.2.7, and 4.2.9). Therefore, both (R/A and untreated) rflaA:Ova proteins had comparable immune modulatory effects. In general, disruption of IgE-reactive conformational B cell epitopes of allergens is a technique to increase safety by reducing the risk of IgE-mediated side effects during SIT, whereas T cell epitopes (which are mostly linear) are retained (210-212). Hence, fusion of a hypoallergenic allergen variant to the TLR5-ligand flagellin combines the immune modulating capacities of flagellin fusion proteins with the increased safety attributed to partially defolded allergens and can therefore be considered an interesting candidate for safer immunotherapy.

5.2.10 The immune modulating properties of rflaA:Ova are probably mediated by a four step mechanism

In order to explain the cellular mechanism underlying the strong activation of mDC observed upon stimulation with rflaA:Ova fusion proteins and its effect on co-cultured T cells, a four step mechanism is proposed (Figure 74): (i) fusion of flagellin to allergens efficiently targets the allergens to TLR5-expressing cells (such as mDC), (ii) the fusion protein is efficiently internalized by DC via TLR5-dependent and/or –independent mechanisms, which probably is influenced by biophysical characteristics (such as the molecular weight or aggregation state), which leads, by a yet unknown mechanism, to a (iii) strong MyD88-dependent TLR5 up-regulation which in turn potentiates DC-activation and cytokine secretion via additional binding of flagellin fusion proteins to TLR5. (iv). This cell activation and induction of IL-10 secretion mediated by TLR-signaling leads to the suppression of Th1 and Th2 cytokine secretion observed in the *in vitro* co-culture experiments.

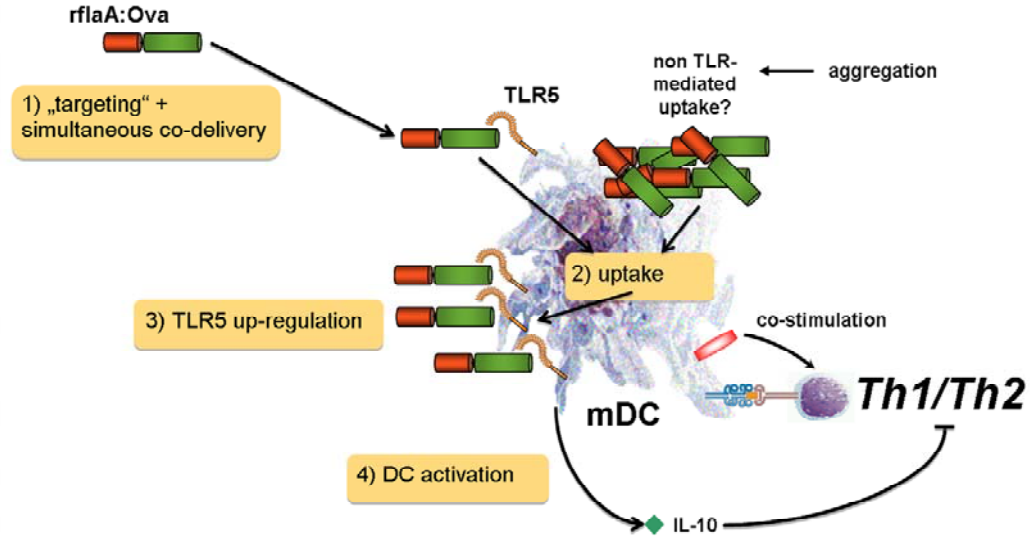


Figure 74: The immune modulating properties of rflaA:Ova may be mediated by a four step mechanism. For detailed information see 5.2.10.

5.3 Evaluation of rflaA:Ova *in vivo*

5.3.1 The model of Ova-induced intestinal allergy is suitable for vaccine testing

Besides the observed effects in cell culture assays, it has to be considered that effects of flagellin stimulation are cell type specific and depend on the experimental system applied (213,214). Moreover, *in vivo* the immune response to adjuvants such as flagellin is orchestrated by the cytokine network and the involvement of several immune and effector cells. Therefore, the immune modulating potential of rflaA and rflaA:Ova were further analyzed using the mouse model of Ova-induced intestinal allergy (see 3.3.1, Burggraf *et al., in revision*).

The *in vivo* mouse model applied in this study proved to be highly suitable for the testing of flagellin A and the flagellin A-containing vaccine candidate. Sensitization to Ova can easily be achieved by just two i.p.-injections of Ova with aluminium hydroxide and the continuous challenge with Ova-containing food pellets is suitable to induce intestinal allergy. After sensitization and subsequent challenge mice showed distinct signs of illness such as weight loss, soft faeces, ruffed fur, and reduced mobility. These symptoms (core body temperature, body weight and food uptake) can be monitored easily (see 4.3.2.1 and 4.3.2.2). The observed drop in core body temperature during the acute phase of the disease indicates that the intestinal allergy induced by continuous Ova-feeding also results in a pronounced systemic reaction. Finally, experience gained from three independent vaccination studies

(twice prophylactic vaccination plus one therapeutic trial) showed the model of Ova-induced intestinal allergy to deliver stable and reproducible results. Scoring clear and reproducible disease associated symptoms is a prerequisite for testing vaccine candidates for their protective capacity *in vivo*. Therefore, the different constructs generated in this study were tested for their prophylactic and therapeutic potency using the above described model of Ova-induced intestinal allergy.

5.3.2 Prophylactic vaccination with rflaA:Ova protects against intestinal allergy

Prophylactic vaccination with the fusion protein but not with the single components either provided alone or as an equimolar mixture was sufficient to prevent allergy (see 4.3.2.1 and 4.3.2.2). Here, both intraperitoneal and intranasal application routes were shown to be effective in preventing the establishment of allergy (see 4.3.2.8). This protective effect was accompanied by the prevention of weight loss, drop in core body temperature, soft faeces, and a strong reduction of disease associated symptoms such as ruffed fur and reduced mobility compared to either non-vaccinated, or Ova-, rflaA-, and rflaA plus Ova-vaccinated groups. Furthermore, mice that were treated by prophylactic vaccination with the fusion protein showed a constant food uptake, whereas Ova-, rflaA, and rflaA plus Ova-vaccinated animals displayed dramatic decreases in food uptake on days 4.5 to 6.5 of Ova-pellet challenge during the most acute phase of the disease (see 4.3.2.1). Finally, in accordance with the strongly reduced symptoms, prophylactic vaccination with the fusion constructs was shown to reduce CD4 and CD8 T cell activation (see 4.3.2.5) as well as Th2 cytokine secretion in intestinal homogenates (see 4.3.2.6) compared to non-vaccinated of rflaA plus Ova-vaccinated groups.

Although intranasal administration of the fusion protein was also shown to completely protect against allergic symptoms and was even more potent in reducing Ova-specific IgE production than i.p.-injection (see 4.3.2.8) it was decided to focus on i.p.-injection. Intranasal injection is performed three times within nine days under anaesthesia which leads to a significant weight loss. This anaesthesia-induced weight loss causes problems in the intestinal allergy model, since the combined weight loss induced by anaesthesia and Ova-pellet challenge would become that large that PBS-vaccinated control mice would have to be killed due to animal welfare regulations.

5.3.3 Prophylactic vaccination prevents T cell activation

To clarify the mechanism of this protection extensive cellular analyse were performed. Investigation of T_{reg} frequencies in spleens and mesenteric lymph nodes (which were in accordance with the expected frequencies known from literature) during the acute phase of the allergy did not reveal pronounced differences between the different treatment groups (see 4.3.2.4). Hereby, splenic T_{reg} frequencies refer to systemic, whereas MLN T_{reg} frequencies refer to local immune responses. Therefore, at the time points investigated (days 4.5 to 6.5) the protection observed in rflaA:Ova-vaccinated animals cannot be explained by an altered T_{reg} frequency.

Despite the lack of T_{reg} induction, both spleen and mesenteric lymph node derived T cells displayed a reduced T cell activation as determined by high CD62-L expression in rflaA:Ova-vaccinated animals compared with both, control and other vaccination groups (see 4.3.2.5). This reduction of both, CD4 and CD8 T cell activation, is in accordance with the suppression of Th2 cytokine secretion in intestinal homogenates and the strongly reduced clinical symptoms in animals vaccinated with the fusion protein (see 4.3.2.1). Besides the effects of rflaA:Ova-vaccination on T cells, no striking differences in B cell and DC activation were observed during the acute phase of the disease (see 4.3.2.5). Taking into consideration the protective effect observed after prophylactic vaccination with the fusion protein it may be stated that the chosen time point for investigating B cell and DC function, namely during the acute phase of the disease might be too late to monitor effects in these cell types. Activation of DC and the resulting differentiation of B cells into Ova-specific plasma cells likely take place at earlier time points after the vaccination.

Since no differences in T_{reg} frequencies were observed between the different treatment groups IL-10 secretion was determined in serum samples. Here, a strong induction of IL-10 (ranging from 1600 to 6700 pg/ml) was only detectable in four out of eight rflaA:Ova-vaccinated mice and median IL-10 levels were not different from either non-vaccinated or rflaA plus Ova-vaccinated animals. However, the reduced T cell activation observed in spleens and MLN of rflaA:Ova-vaccinated animals needs to be evaluated at an earlier time point. In line with this, the *in vitro* experiments performed in this thesis have clearly shown that rflaA:Ova is able to induce IL-10 secretion from myeloid DC (see 4.2.2) resulting in the suppression of naïve as well as Th2-biased CD4 TC (see 4.2.9, 4.2.12, and 4.2.14). *In vivo* IL-10 was shown to suppress IL-4-mediated class-switching to IgE (215). Therefore, *in vivo* application of the fusion protein will supposably also result in an IL-10-mediated suppression of Ova-specific T cell differentiation and therefore prevent the establishment of intestinal allergy.

In accordance with this hypothesis, both rflaA and rflaA:Ova were shown to activate splenic DC 24 h post i.p.-administration (see 4.3.1), a finding recently confirmed by Braga and

coworkers (216). Despite the problems encountered when determining cytokine levels in murine sera using multiplex ELISA (strong heterogeneity between animals within the groups), low doses of rflaA:Ova were shown to induce a variety of cytokines, including immunosuppressive IL-10, flagellin responsive cytokines such as IL-18 and IL-22, and Th1 promoting IFN-γ (see 4.3.1). These results suggest that vaccination with rflaA:Ova might induce a more balanced immune response towards Ova, compared to Ova-administration without flagellin.

Moreover, the rflaA:Ova fusion protein was shown to induce high amounts of IL-6 from mDC *in vitro* (see 4.2.2 and 4.2.12). Stimulation with lower concentrations of rflaA:Ova-induced IFN-γ secretion in mDC:T cell co-cultures stimulated with rflaA:Ova (see 4.2.12). IL-6, IFN-γ, and IL-4 are known to be potent inducers of antibody production: IFN-y promoting the induction of IgG2a and the suppression of IgE subclasses, IL-6 displaying an important role in Th17 induction, whereas IL-4 (which was not detectable in the sera analyzed) promotes IgG1 and IgE production (217-219).

The strong secretion of IL-6 from rflaA:Ova stimulated mDC raised the question whether the application of the fusion protein *in vivo* might result in Th17-dominated immune responses that could potentially result in detrimental autoimmune responses. However, only low amounts of IL-23, another cytokine needed for Th17 differentiation were detected from stimulated mDC (data not shown). Moreover, no IL-17 production was detectable in neither sera nor intestinal homogenates of any of the investigated groups (data not shown). These results show that the application of Ova in context of fusion to flagellin did not result in exaggerated Th17 responses. The lack of Th17-biased T cell responses upon flagellin fusion protein stimulation improves the safety profile of such vaccines.

5.3.4 Prophylactic vaccination with rflaA:Ova enhances IgG2a and suppresses IgE production

In accordance with the cytokine production observed 24 h post i.p.-vaccination (see 5.3.3, IL-10, IFN-γ production in sera) only prophylactic vaccination with rflaA:Ova, but not with the mixture of flagellin plus Ova, Ova, or rflaA alone suppressed Ova-specific IgE-production (see 4.3.2.7). Moreover, a distinct induction of Ova-specific IgG2a antibodies was only observed when flaA and Ova were covalently fused (see 4.3.2.7 and Figure 75), indicating that B cell differentiation and function were strongly influenced by vaccination with the fusion protein.

In accordance with the *in vitro* results obtained in the present study this suggests that targeting the same effector cell with the fusion protein had superior immune modulating capacity compared to the mixture of both components. In another study, the co-application of

flagellin and the major bee allergen phospholipase A2 (PLA2) as a mixture was shown to induce PLA2-specific IgG2a antibody production in mice (34). This induction of IgG2a antibodies by flagellin may also apply for the induction of Ova-specific antibodies if Ova is recognized by the immune system in the context of fusion to flagellin. In accordance with this assumption an induction of Ova-specific IgG2a antibodies was only observed in rflaA:Ova-vaccinated animals (see 4.3.2.7).

Although rflaA:Ova-vaccinated mice were completely protected against intestinal allergy, no differences in neither splenic nor lymph node T_{reg} frequencies (see 4.3.2.4), or proliferation of *ex vivo* purified splenic or MLN-derived CD4 T cells upon re-stimulation with ovalbumin (see 4.3.2.3) were observed. Here, the observed alteration in B cell responses upon Ova-pellet challenge might explain this protection. The reduced Ova-specific IgE-production in conjunction with both, the strong induction of Ova-specific IgG2a-antibodies and the reduced CD4 and CD8 T cell activation is likely to explain the protective effect.

Such strongly decreased IgE-levels result in reduced basophil degranulation induced by allergen-mediated IgE-crosslinking upon contact with the allergen (Figure 75). Simmilar results were obtained by Albrecht and co-workers using a recombinant modified vaccinia virus Ankara expressing Ova (MVA-Ova) (220). Prophylactic vaccination with MVA-Ova lead to a reduced and delayed production of Ova-specific IgE antibodies and conferred protection against allergic sensitization in 50% of vaccinated animals. In accordance with the results presented in this thesis, Ova-specific IgG1 titers were unchanged upon vaccination, whereas Ova-specific IgG2a titers were strongly increased in MVA-Ova-vaccinated mice, suggesting a Th1-biased immune response (220).

In addition, high levels of IgG2a may neutralize the allergen and act as so-called blocking antibodies (221) avoiding allergic reactions (Figure 75). In accordance with the results presented in this study Schmitz et al. (222) reported the protection against cat allergy observed upon vaccination of mice with Fel d 1 coupled to the virus like particles, to be independent of the induction of regulatory T cells or the effector function of CD4 T cells. Further analysis by Schmitz and co-workers lead to the conclusion, that inhibitory signaling of Fel d 1-specific IgG antibodies via FcyRIIb was sufficient to confer protection upon challenge with the allergen (222). However, the mechanism underlying the protective effect of allergen-specific IgG2a is still not fully understood.

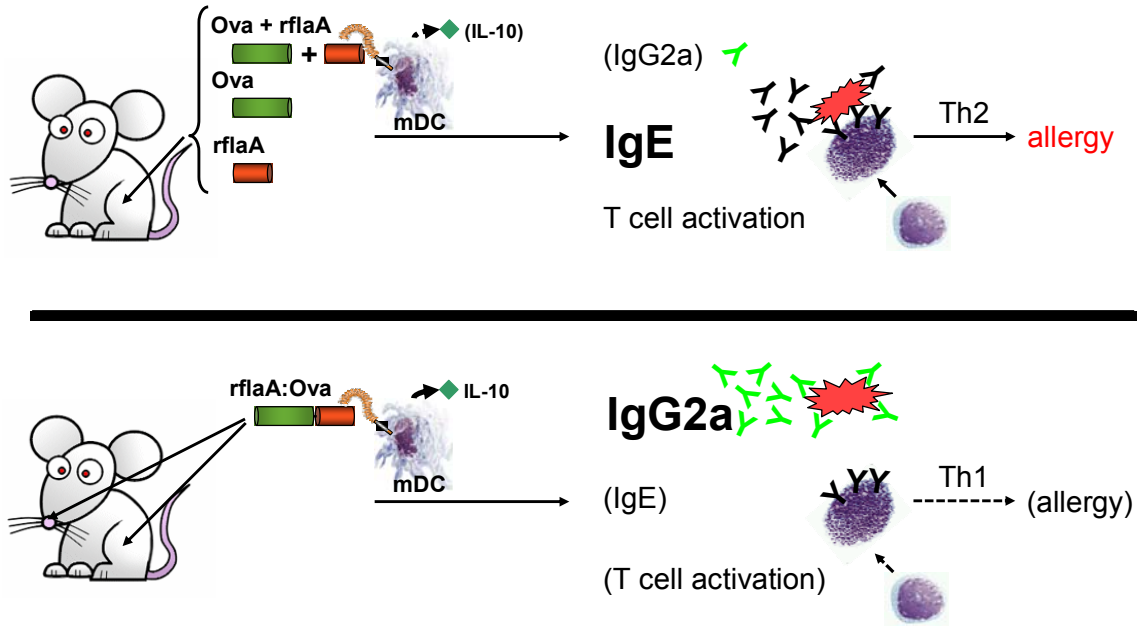


Figure 75: Prophylactic vaccination with rflaA:Ova but not with rflaA, Ova, or rflaA + Ova prevents allergic sensitization. For detailed information see 5.3.3 and 5.3.4.

Interestingly, the mixture of flagellin and Ova did not have a similar protective effect as the fusion protein, leading to a stronger weight loss, temperature drop and allergic symptoms (see 4.3.2.1 and 4.3.2.2). Furthermore, vaccination with both components did neither suppress Ova-specific IgE production nor induce significant levels of Ova-specific IgG2a antibodies (see 4.3.2.7). Although there is currently no experimental explanation for this effect, it is speculative that application of the mixture of both components may result in an "adverse adjuvant effect" of flagellin. In this scenario, during vaccination *in vivo* both components are likely internalized by different cells or cell types. Ova taken up by APC without flagellin would induce Th2 T cell differentiation, resulting in IgE production and mediator release from sensitized mast cells and basophils. By contrast flagellin would activate TLR5 expressing cells such as DC and epithelial cells, resulting in strong cytokine secretion and an even enhanced recruitment of effector cells. Therefore, this combination of mast cell activation and enhanced cell recruitment may result in bystander activation of Ova-specific cells and therefore the aggravated symptoms observed upon vaccination with the mixture of flagellin and Ova (see 4.3.2.1 and 4.3.2.2).

The antibody induction by the fusion protein is of particular interest, since in human clinical trials successful desensitization is accompanied by mostly unchanged levels of allergen-specific IgE, whereas serum concentrations of other antibody subclasses, such as IgG4 increase (92,223). Therefore, as the flagellin fusion protein investigated in this study was both capable of increasing allergen-specific IgG2a-levels and preventing the induction of

allergen-specific IgE, such constructs may possess potential for the treatment of allergies in humans.

Similar protective effects of vaccination with fusion proteins containing flagellin and various viral and bacterial proteins were reported by several other groups. Administration of these constructs resulted in strong antibody responses directed against the fused antigen that protected against subsequent challenge with the respective pathogen (142,144,145,196,197,224). In line with the results presented in this thesis, immunization of C57BL/6 mice with a *Salmonella* flagellin C-Ova fusion protein was shown to result in both the induction of Ova-specific IgG1 and IgG2a antibodies and antigen-specific T cell responses (145). Moreover, intramuscular immunization of African green monkeys with a fusion protein consisting of *Pseudomonas* flagellins A and B and a *Pseudomonas* antigen OprI induced robust IgG responses protecting against *Pseudomonas* infection via extensive complement activation (197,225). Finally replacement of the hypervariable region of Salmonella flagellin C with the influenza HA globular head resulted in a highly protective vaccine inducing high titers of anti HA antibodies (196). Interestingly, so far none of the investigated flagellin fusion proteins induced significant increases in antigen-specific IgE antibody production (206).

5.3.5 Therapeutic vaccination with the different constructs is not able to reverse an established allergic response

Finally, a preliminary therapeutic vaccination study was performed to test whether treatment with the different constructs was sufficient to reverse an established allergic response (see 4.3.3). In the application scheme used, neither treatment with Ova, rflaA, rflaA plus Ova, nor the covalent fusion protein rflaA:Ova was sufficient to prevent weight loss, temperature drop, or soft faeces (see 4.3.3.1 and 4.3.3.3). However, compared to control groups rflaA:Ova treated mice displayed a less pronounced softness of faeces and therefore a slightly but significantly reduced clinical symptom score (see 4.3.3.1). Moreover, rflaA:Ova-treated animals showed a constant food uptake (see 4.3.3.2), indicating at least some protective effect of therapeutic vaccination with rflaA:Ova. In accordance with the results obtained for the prophylactic vaccination study (see 4.3.2.5), rflaA:Ova-treated animals displayed a reduced CD4 and CD8 T cell activation as determined by high levels of CD62-L expression. However, this reduced T cell activation alone was not sufficient to prevent intestinal allergy upon Ova-pellet challenge. In accordance with the different constructs' inability to suppress weight loss or temperature drop, no increase of Ova-specific IgG2a-levels or suppression of Ova-specific IgE antibodies were observed in sera of mice subjected to therapeutic vaccination (see 4.3.3.7). Taken together, data obtained from cellular analysis of both

therapeutic and prophylactic vaccination studies showed (i) unaltered frequencies of regulatory T cells in the different treatment groups (see 4.3.3.5), and (ii) no significant changes in B cell and DC activation status (see 4.3.2.5 and 4.3.3.6). Therefore, these results suggest that the observed changes in serology and the local reduction of Th2-cytokines in intestinal homogenates, supported by the reduced T cell activation observed upon vaccination with the fusion protein might be responsible and sufficient to explain the observed protection against intestinal allergy.

It is likely that the used scheme for therapeutic intervention with only two i.p-injections relatively short after Ova-sensitization is not optimal to interfere with the established Th2-biased immune response. In accordance with the results obtained in the therapeutic vaccination study in this thesis, Bellinghausen and co-workers observed that the transfer of IL-10 treated DC into BALB/c mice immunized with Ova in alum was not sufficient to influence airway inflammation and IgE-production, even though the treatment suppressed T cell proliferation and cytokine secretion (83). These results suggest that established Th2 responses induced by immunization with Ova in Alum are less susceptible to inhibition by both IL-10 treated DC and (IL-10 inducing) flagellin fusion proteins (83).

In summary, therapeutic vaccination with the fusion protein was able to reduce disease symptoms but not sufficient to induce Ova-specific IgG2a antibodies, or suppress IgE-production or symptoms. Here, further optimization is required to improve therapeutic efficacy.

5.4 Flagellin-containing fusion proteins are promising vaccine candiddates for the prevention of allergies

Despite the broad investigation of flagellin containing fusion proteins as vaccines for different bacterial and viral pathogens, for allergic diseases caused by pathologic, Th2-driven immune responses the application of flagellin fusion proteins has not yet been investigated so far.

Currently, various bacteria (either as live or heat killed preparations) are investigated for their potential to treat or protect against allergies. For example, in sensitized mice the intranasal co-administration of *L. lactis* and *L. plantarum* with the birch pollen allergen Bet v 1 (both as mixture and as recombinant Bet v 1-expressing strains) was shown to induce protective Th1-responses characterized by high levels of Bet v 1-specific IgG2a antibodies and IFN-γ production (226,227). Consistent with these results, in Ova-sensitized mice sublingual vaccination with one defined bacterial component, the TLR9-agonist CpG also induced Th1-dominated immune responses, characterized by high levels of IgG2a antibodies and IFN-γ secretion (228). Sublingual administration of the nontoxic cholera toxin B subunit with Ova

was shown to result in depletion of Ova-specific effector T cells in peripheral lymph nodes, mediated by T_{reg} dependent increases in apoptosis rates (229). In contrast, the present work describes a fusion protein consisting of the TLR5-ligand flagellin and ovalbumin which strongly activates myeloid DC *in vitro*, resulting in mDC activation and cytokine secretion. Among the cytokines secreted anti-inflammatory IL-10 was shown to suppress both Th1 and Th2 cytokines not only from naïve CD4 T cell *in vitro*, but also from Th2-biased *ex vivo* purified Ova-specific CD4 T cells. *In vivo* prophylactic vaccination with rflaA:Ova was shown to prevent allergic sensitization in a model of Ova-induced intestinal allergy. Here, protection against allergy was independent of T_{reg} induction but accompanied by a distinct induction of Ova-specific IgG2a-antibodies, while IgE-production was suppressed. In accordance with the strongly reduced symptoms, rflaA:Ova vaccination was shown to result in a reduced CD4 and CD8 T cell activation which was accompanied by a reduction of Th2-cytokine production in intestinal homogenates. Therefore, depending on the component used for vaccination different mechanism are likely to account for the observed preventive effects. Flagellin acting via IL-10 secretion by DC, whereas CpG induces Th1-biased immune activation and cholera toxin B results in IL-10 independent depletion of effector T cells.

6. Summary

Currently, there is a need for new therapeutic strategies for the treatment of type I allergies, since specific immunotherapy is accompanied by a rather high risk of side effects. The aim of this thesis was to investigate whether flagellin-allergen fusion proteins can be considered as vaccines to improve the specific immunotherapy of Th2-biased allergic diseases. Flagellin is a Toll-like receptor 5 (TLR5) ligand and flagellin stimulation results in a more Th1-biased activation of TLR5 expressing antigen presenting cells (APC). Therefore, the simultaneous co-delivery of flagellin and allergen to TLR5 expressing APCs in a fusion protein may modulate Th2-dominated allergen-specific immune responses.

Selecting ovalbumin (Ova) as a model allergen, the immune modulating properties of flagellin fusion proteins were evaluated (i) *in vitro* using murine DC subsets as well as naive and *ex vivo* purified Ova-specific CD4 TC and (ii) *in vivo* using a model of Ova-induced intestinal allergy. Therefore, recombinant (r) *Listeria monocytogenes* flagellin A (rflaA), rOva, and the covalent fusion protein of rflaA and Ova (rflaA:Ova), as well as fusion proteins containing major peanut (Ara h 2) and peach allergens (Pru p 3), rflaA:Ara h 2 and rflaA:Pru p 3, respectively, were generated by cDNA assembly. All proteins were purified in milligram amounts from *E. coli* by chromatographic methods, displayed considerable formation of secondary structure elements, and negligible endotoxin contamination. All fusion proteins were able to activate TLR5, as determined by TLR5 transgenic HEK293 reporter cells.

Using *in vitro* differentiated, bone marrow-derived mouse myeloid DC (mDC) from BALB/c and C57BL/6 mice, the immune modulating properties of rflaA:Ova, rflaA:Pru p 3, and rflaA:Ara h 2 were shown to be superior compared to an equimolar mixture of both components. All fusion proteins were shown to efficiently activate mDC by inducing CD40 expression and IL-1ß and IL-6 secretion. Moreover, rflaA:Ova, used as model protein, strongly enhanced mDC TLR5 expression and IL-10 secretion in comparison to an equimolar mixture of both components. In contrast, plasmacytoid DC (pDC) did not show comparable TLR5 expression, cell activation, or considerable cytokine secretion upon stimulation with the different constructs. In co-culture experiments using mDC with Ova-specific CD4 T cells, rflaA:Ova dose-dependently repressed the Ova-induced secretion of Th1 and Th2 cytokines. Furthermore, rflaA:Ova was also able to suppress Ova-induced Th1 and Th2 cytokine secretion from *in vivo* differentiated Th2-biased CD4 T cells. Remarkably, flagellin fusion proteins slightly tend to form high molecular aggregation, due to intermolecular disulfide bonds between allergen molecules. To address the influence of aggregation on the immune modulating properties a partially defolded rflaA:Ova variant was generated by reduction and alkylation (R/A). rflaA:Ova R/A retained the capacity to activate mDC, induce cytokine

secretion, up-regulate TLR5 on mDC, and stimulate CD4 T cells. Using mDC derived from MyD88$^{-/-}$ mice, the suppression of IFN-γ secretion was shown to be rescued, whereas the rflaA:Ova-induced secretion of IL-6 and IL-10 from mDC was abolished. Most likely the superior immune modulating effects are due to a targeting of antigen to TLR5^{+} APC, an enhanced uptake by TLR5^{+}CD11b^{+}CD11c^{+}B220^{-} mDC, and subsequent strong TLR5-upregulation leading to an enhanced TLR-mediated cell activation.

Moreover, the immune modulating properties of rflaA and rflaA:Ova were tested *in vivo* using a murine model of Ova-induced intestinal allergy. Prophylactic vaccination with the rflaA:Ova fusion protein in contrast to the single components or the mixture of both proteins, was sufficient to prevent allergic sensitization while inducing more Th1-dominated immune responses. This protection against intestinal allergy was characterized by a suppression of phenotypic symptoms, weight loss, soft faeces, and core body temperature drop, as observed in control groups. In accordance with the reduction of clinical symptoms fusion protein-vaccinated mice displayed a reduced T cell activation as well as reduced levels of Th2 cytokines in intestinal homogenates compared to either untreated animals or animals treated with the mixture of both components. Among the different treatment groups no differences in T_{reg} frequency were observed. Furthermore, application of rflaA:Ova was shown to suppress Ova-specific IgE production, while inducing Ova-specific IgG2a antibodies, which might act as blocking antibodies.

In a therapeutic vaccination approach, neither treatment with rflaA, rflaA plus Ova, nor rflaA:Ova was sufficient to prevent weight loss, or temperature drop in the used vaccination scheme. However, rflaA:Ova-vaccinated mice displayed slightly reduced clinical symptom scores due to reduced softness of faeces and a constant food uptake when compared to control groups. In this experimental setting no repression of Ova-specific IgE production and no induction of IgG2a production were observed in any of the investigated treatment groups. Here, optimization of the therapeutic vaccination scheme is required to improve therapeutic effectiveness of the fusion protein.

Taken together the results demonstrate that fusion of allergens to TLR5-ligand flagellin resulted in constructs potently modulating DC-controlled immune responses *in vitro* and preventing allergic sensitization *in vivo*. These results suggest that bacterial components and especially TLR-ligands with their ability to activate the host immune system are potent immune modulators which hold great potential for the treatment of allergic diseases.

7. Outlook

The results presented in this thesis showed fusion proteins containing flagellin and allergens to have superior immune modulating properties compared to the single components either provided alone or as a mixture. To further investigate the potential of flagellin fusion proteins additional experiments are necessary. These studies should aim at elucidating the mode of action of flaA-based vaccines both *in vitro* and *in vivo*, as well as the optimisation of therapeutic vaccination, and the application of clinically relevant allergens and corresponding allergy models.

1. Investigation of the molecular mechanism

The superior immune modulating properties of the rflaA:Ova fusion protein were hypothesized to be caused by TLR-mediated cell activation, in combination with an increased protein uptake, and likely to be affected by protein aggregation. To better understand the impact of protein aggregation on the observed cell activation analytical methods need to be established in order to quantify both, the degree of aggregation and the size of the individual aggregates. Moreover, the speculated enhanced uptake of the fusion protein by mDC should be experimentally verified by performing time-dependent uptake studies (e.g. using fluorescence marker coupled proteins). In this context, to investigate the impact of the protein structure on protein uptake, hydrophobicity tests should be included. Moreover, antigen processing (rflaA:Ova vs rOva) can be monitored by lysosomal digestion experiments and analysis of Ova-derived peptides (sequence and time-dependent generation). Additionally, co-culture experiments should be repeated using mDC derived from IL-10 deficient mice (BALB/c background) in order to confirm the influence of rflaA:Ova-induced IL-10 secretion on the observed immune modulation. Moreover, to confirm the contribution of TLR5 to the observed effects mDC-derived from TLR5$^{-/-}$ mice should be stimulated with the fusion protein and checked for cell activation and cytokine secretion. To clarify whether fusion protein stimulation in allergic patients might result in “cross-activation” of DC by allergen-specific IgE (bound to FcεRI on DC) and TLR5, murine mDC could be passively sensitized *in vitro* using Ova-specific IgE and subsequently stimulated with rflaA:Ova. So far the immune modulating capacities of rflaA:Ova were only investigated using mDC either alone or co-cultured with Ova-specific CD4 T cells. To further understand the influence of flagellin fusion proteins on the immunological network the modulation of other TLR5-expressing cell types (e.g. macrophages, mast cells, or T cells) should be investigated.

2. Mechanism of rflaA:Ova-mediated protection in vivo

To better understand the contribution of Ova-specific IgG2a antibodies induced by rflaA:Ova to the observed protection upon prophylactic vaccination *in vivo* serum transfer experiments should be performed. Therefore, complement-inactivated sera obtained from mice vaccinated with the fusion protein should be transferred into Ova-sensitized but non-vaccinated animals. These experiments will provide further evidence whether allergen-specific IgG antibodies contribute to the observed protective effect. Moreover, since the Fc_yRIIb receptor was shown to mediate allergen-specific inhibitory effects of Fel d 1-specific IgG2a antibodies in sensitized mice (222) Fc_yRIIb-deficient mice should also be included in future studies. Furthermore, IL-10 deficient mice may be used to investigate whether IL-10 induction from mDC observed *in vitro* might contribute to the rflaA:Ova mediated protection *in vivo*. So far, no or only very weak anti-flagellin immune responses, that could neutralize flaA-derived vaccines *in vivo*, have been reported (158,195,230). In line with these results, to better characterize the overall immune response sera of vaccinated mice should be checked for rflaA-specific antibody responses. These antibodies could impair vaccine efficacy, by vaccine neutralization and decreased TLR5-activation. Additionally, Ova-specific IgA titers should be determined to investigate the influence of the mucosal adjuvant flagellin on IgA production. To confirm the reduced cell activation in rflaA:Ova-vaccinated animals proliferation assays should be repeated and cell supernatants should be analyzed for their cytokine profile. Moreover, a time-dependent analysis of the humoral immune response, T cell activation, T cell proliferation, and cellular parameters upon vaccination and Ova-pellet challenge should allow more detailed insights into the mechanism of protection. In the present study, the preventive effect of rflaA:Ova-vaccination on intestinal allergy was monitored during the acute phase during, and at the end of Ova-pellet challenge. The duration of the preventive effect upon continuous or repeated (re-)challenges with Ova-containing pellets might be addressed. Although no adverse effects were observed upon application of rflaA:Ova *in vivo,* further studies related to safety and efficacy should be performed.

3. Optimization of vaccination schedule

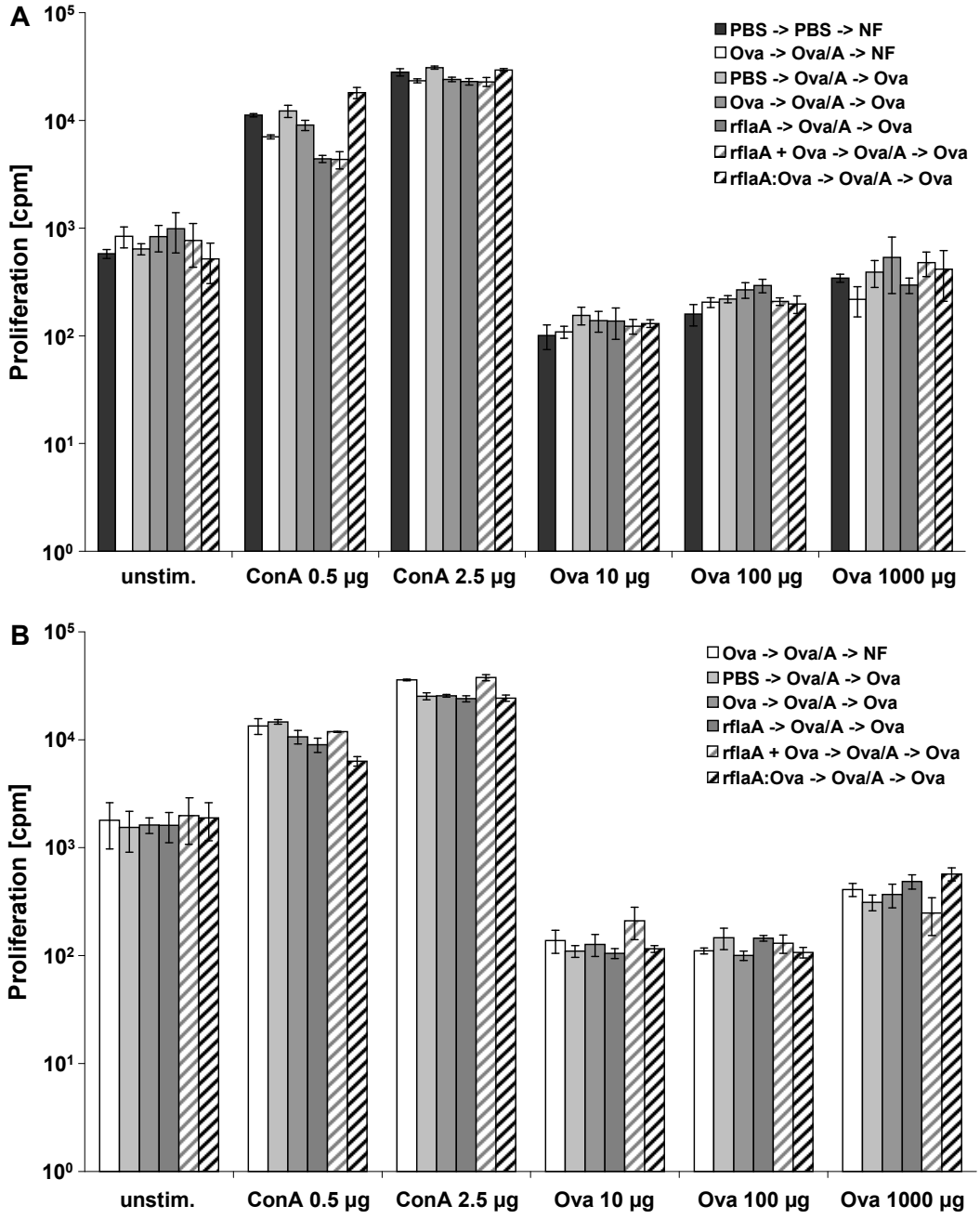
An initial therapeutic treatment approach with rflaA:Ova was shown to be insufficient to cure Ova-induced intestinal allergy. Here, it is likely that the vaccination scheme used for therapeutic intervention (two i.p-injections two weeks after Ova-sensitization) is not optimal to interfere with the established Th2-biased immune response. Therefore, increased numbers of immunizations with optimized dosages of fusion protein, or intranasal application which was shown to result in an even more efficient suppression of Ova-specific IgE production

(see 4.3.2.8) might increase therapeutic potency of the fusion construct. Furthermore, optimization of the time interval between sensitization and first vaccination and the time interval between single vaccinations might strongly increase therapeutic efficacy.

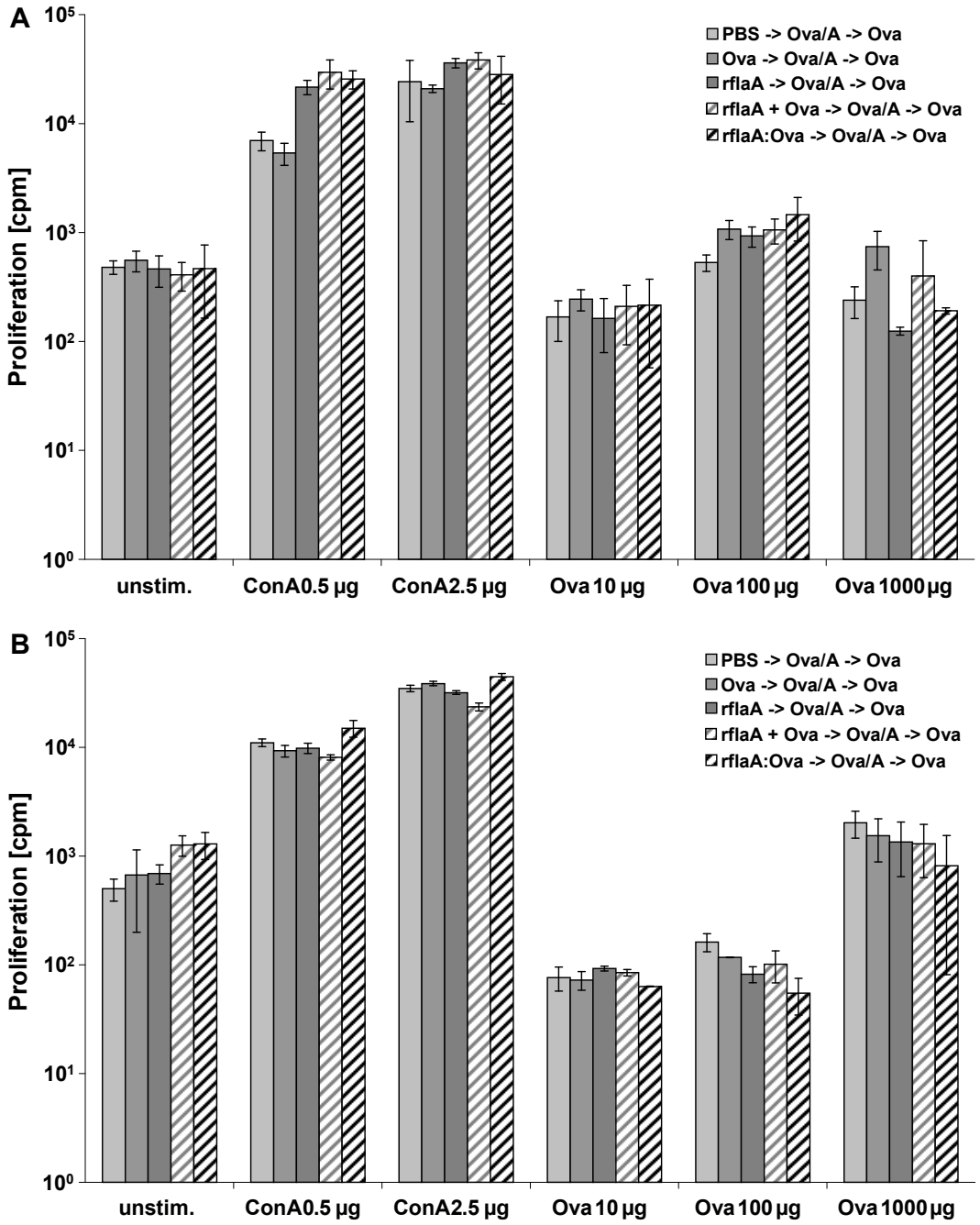
4. Usage of clinial relevant allergens

Finally, to further characterize the potency of flagellin containing fusion proteins, constructs consisting of *Listeria monocytogenes* flaA and clinical relevant food (Pru p 3 and Ara h 2), pollen (Bet v 1, the major birch pollen allergen), and house dust mite allergens (Der p 2) should be generated and investigated for their protective capacities *in vivo* using suitable mouse food and inhalant allergy models.

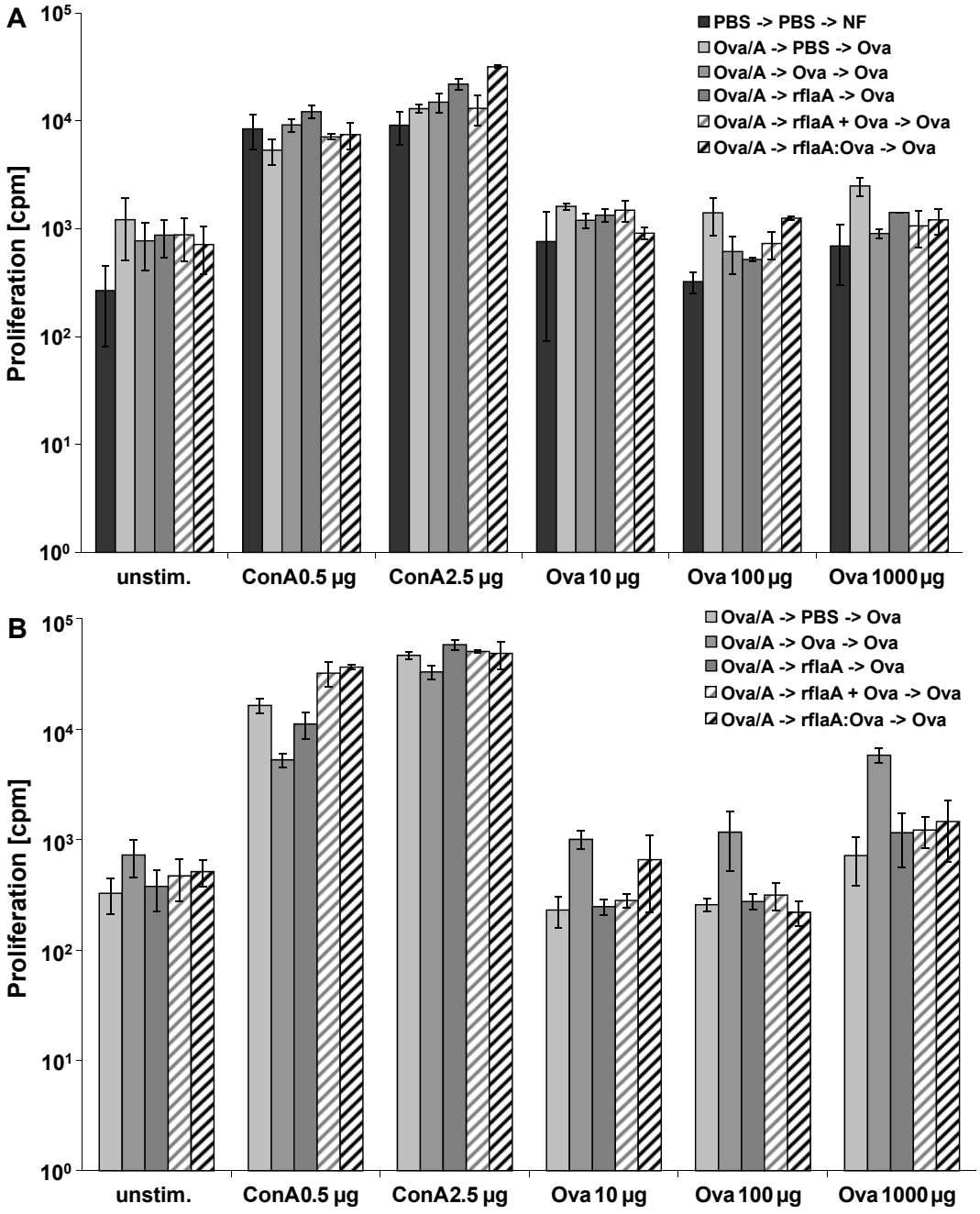
8. Repository figures



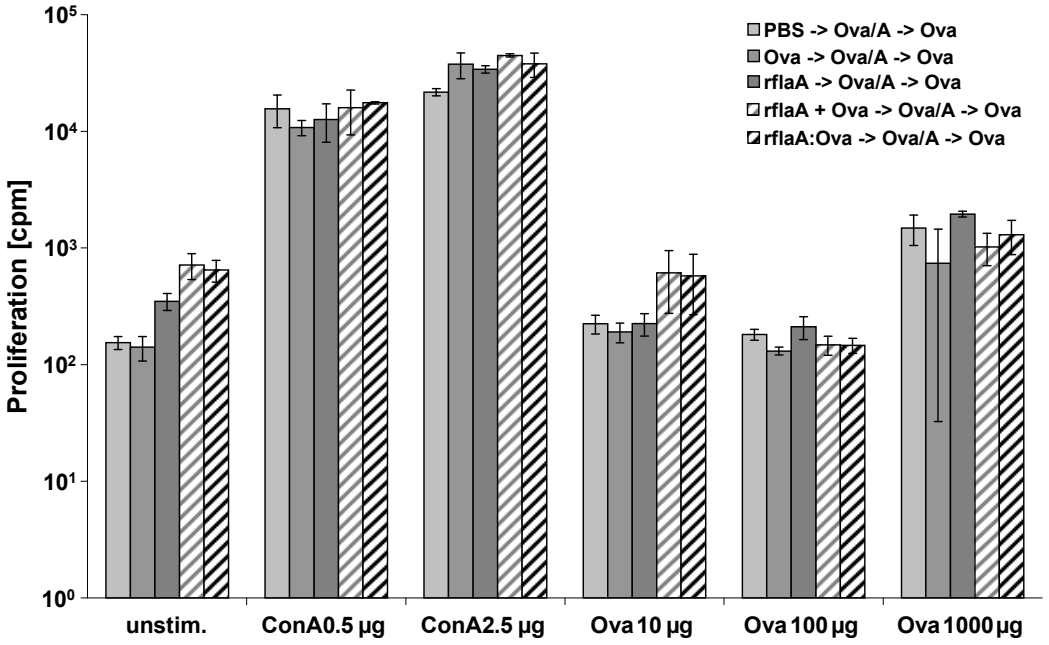
Repository figure 1: Prophylactic vaccination study: Splenic CD4$^+$ T cells show no difference in proliferation upon restimulation with Ova. On day 5.5 (A) and 6.5 (B) of Ova-pellet challenge mice were sacrificed, spleen cells were pooled, and CD4$^+$ TC were isolated from spleens by magnetic cell separation. Purified CD4$^+$ T cells were co-cultured with Mitomycin C treated syngenic APC, restimulated with either ConA or Ova and incubated for 72 h. After 72 h ^{3}H-thymidin was added and cells were incubated for additional 20 h. Proliferation was determined for each day and group (n = 4 mice per group and day).



Repository figure 2: Prophylactic vaccination study: MLN derived CD4$^+$ T cells show diverse proliferation responses upon restimulation with Ova. On day 5.5 (A) and 6.5 (B) of Ova-pellet challenge mice were sacrificed, MLN cells were pooled, and CD4$^+$ TC were isolated from spleens by magnetic cell separation. Purified CD4$^+$ T cells were co-cultured with Mitomycin C treated syngenic APC, restimulated with either ConA or Ova and incubated for 72 h. After 72 h ^{3}H-thymidin was added and cells were incubated for additional 20 h. Proliferation was determined for each day and group (n = 4 mice per group and day).

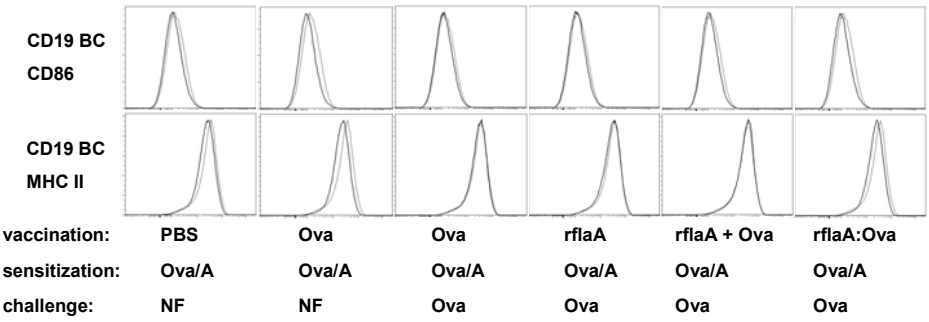


Repository figure 3: Therapeutic vaccination study: Ova-vaccinated animals show increased proliferation rates in splenic CD4$^+$ T cells. On day 5.5 (A) and 6.5 (B) of Ova-pellet challenge mice were sacrificed, spleens were pooled, and CD4$^+$ TC were isolated from spleens by magnetic cell separation. Purified CD4$^+$ T cells were co-cultured with Mitomycin C treated syngenic APC, restimulated with either ConA or Ova and incubated for 72 h. After 72 h ^{3}H-thymidin was added and cells were incubated for additional 20 h. Proliferation was determined for each day and group (n = 3 mice per group and day).



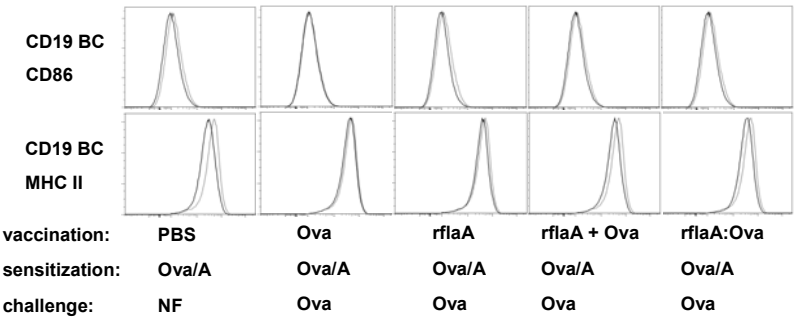
Repository figure 4: Therapeutic vaccination study: MLN derived CD4$^+$ T cells show diverse proliferation responses upon restimulation with Ova. On day 6.5 of Ova-pellet challenge mice were sacrificed, spleens were pooled, and CD4$^+$ TC were isolated from spleens by magnetic cell separation. Purified CD4$^+$ T cells were co-cultured with Mitomycin C treated syngenic APC, restimulated with either ConA or Ova and incubated for 72 h. After 72 h ^{3}H-thymidin was added and cells were incubated for additional 20 h. Proliferation was determined for each day and group (n = 3 mice per group and day).

Day 5.5 spleen			PBS -> NF	Ova -> NF	Ova -> Ova	F -> Ova	F +O -> Ova	F:O -> Ova
TC	CD4	PD-1	++	n. det.	no diff.	-	-	(-)
		CD62-L	no diff.	+	no diff.	+	+	++
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
	CD8	CD62-L	no diff.	+	no diff.	+	+	++
		CD69	n. det.	n. det.	n. det.	n. det.	n. det.	n. det.
BC	CD19	CD40	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD80	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD86	-	-	no diff.	no diff.	(-)	-
		MHC I	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC II	(-)	-	no diff.	no diff.	no diff.	-
DC	CD11c	CD40	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD80	-	-	no diff.	no diff.	no diff.	no diff.
		CD86	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC I	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC II	+	(+)	no diff.	no diff.	no diff.	no diff.



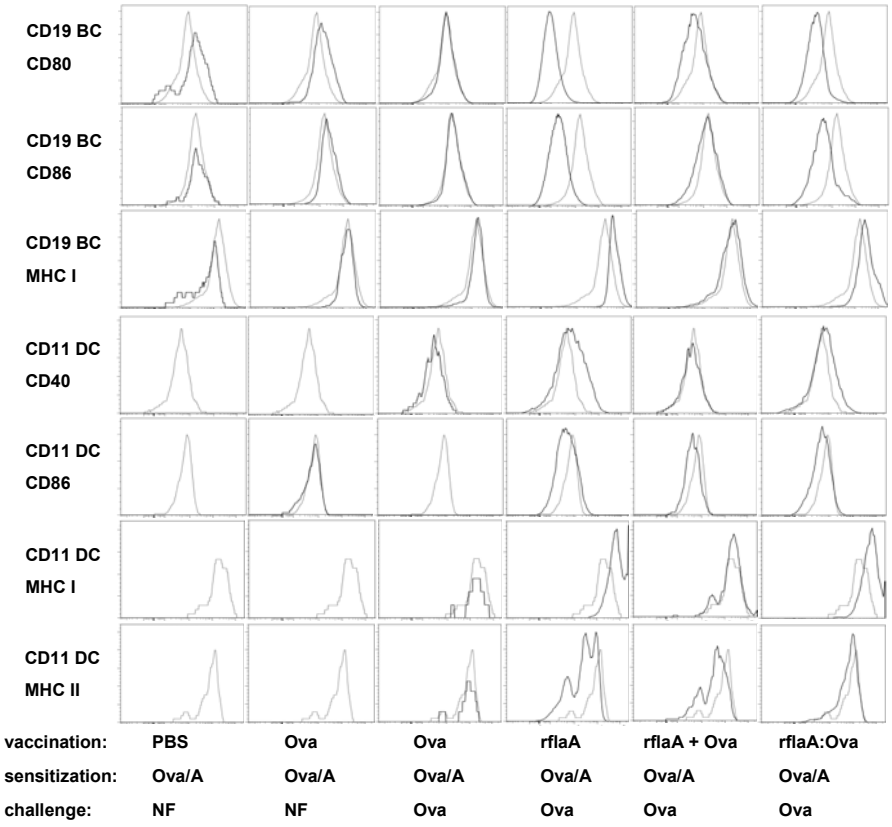
Repository figure 5: Prophylactic vaccination study: Vaccination with rflaA:Ova prevents TC activation. On day 5.5 of Ova-pellet challenge animals were sacrificed, spleens were prepared, and three animals per group were pooled. Subsequently, red blood cells were lysed and cells were stained for the indicated lineage and cell surface markers, expression levels were determined for gated (as indicated) cell populations via flow cytometry and compared to the PBS -> Ova (allergy positive control, grey) group, Abbreviations: *No diff.:* no difference to PBS -> Ova group, *n. det*.: no expression detectable, +: up-regulation, –: down-regulation, (+) / (-): weak up- or down-regulation.

Day 6.5 spleen			PBS -> NF	Ova -> NF	Ova -> Ova	F -> Ova	F +O -> Ova	F:O -> Ova
TC	CD4	PD-1	no diff.	n. det.	no diff.	-	-	-
		CD62-L	n. det.	no diff.	no diff.	++	++	+
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
	CD8	CD62-L	no diff.	n. det.	no diff.	++	+++	+
		CD69	n. det.	n. det.	n. det.	n. det.	n. det.	n. det.
BC	CD19	CD40	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.	+
		CD80	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD86	-	n. det.	no diff.	-	-	-
		MHC I	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC II	-	n. det.	no diff.	(-)	-	-
DC	CD11c	CD40	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD80	-	n. det.	no diff.	0/+	no diff.	(-)
		CD86	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC I	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC II	no diff.	n. det.	no diff.	-	-	no diff.



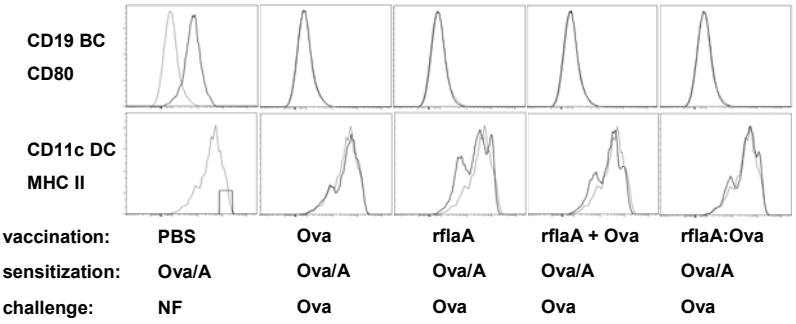
Repository figure 6: Prophylactic vaccination study: Vaccination with flagellin-containing constructs influences T cell activation. On day 6.5 of Ova-pellet challenge animals were sacrificed, spleens were prepared, and three animals per group were pooled. Subsequently, red blood cells were lysed and cells were stained for the indicated lineage and cell surface markers, expression levels were determined for gated (as indicated) cell populations via flow cytometry and compared to the PBS -> Ova (allergy positive control, grey) group, Abbreviations: *No diff.:* no difference to PBS -> Ova group, *n. det.*: no expression detectable, +: up-regulation, –: down-regulation, (+) / (-): weak up- or down-regulation.

Day 5.5 MLN			PBS -> NF	Ova -> NF	Ova -> Ova	F -> Ova	F +O -> Ova	F:O -> Ova
TC	CD4	PD-1	staining failed	staining failed	staining failed	staining failed	staining failed	staining failed
		CD62-L	n. det.	++	++	no diff.	+	+
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
	CD8	CD62-L	+++	+++	+++	no diff.	++	+
		CD69	n. det.	n. det.	n. det.	n. det.	n. det.	n. det.
BC	CD19	CD40	no diff.	no diff.	no diff.	no diff.	no diff.	(-)
		CD69	no diff.	no diff.	no diff.	-	no diff.	(-)
		CD80	+	+	no diff.	--	-	-
		CD86	no diff.	(+)	no diff.	-	(-)	-
		MHC I	(-)	no diff.	no diff.	+	(+)	+
		MHC II	(+)	no diff.	no diff.	(-)	(-)	-
DC	CD11c	CD40	n. det.	n. det.	(-)	++	no diff.	+
		CD69	n. det.	n. det.	no diff.	no diff.	no diff.	(-)
		CD80	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD86	n. det.	no diff.	n. det.	-	-	-
		MHC I	n. det.	n. det.	no diff.	+	no diff.	+
		MHC II	n. det.	n. det.	no diff.	--	-	(-)

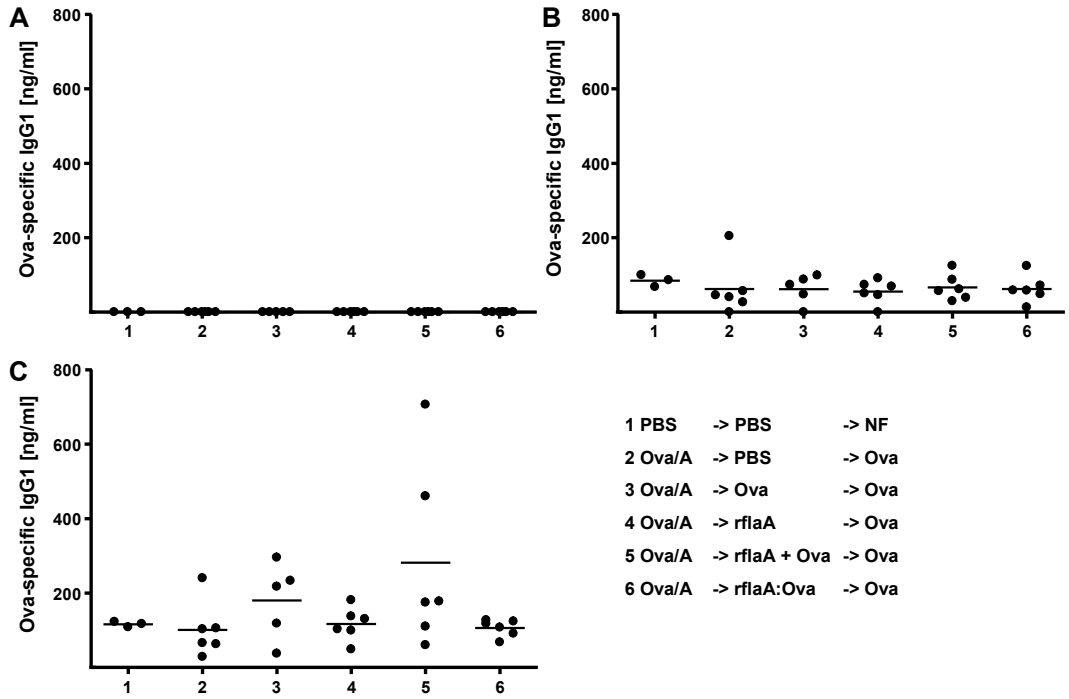


Repository figure 7: Prophylactic vaccination study: Prophylactic vaccination affects many parameters in the mesenteric lymph nodes. On day 5.5 of Ova-pellet challenge animals were sacrificed, mesenteric lymph nodes were prepared, and three animals per group were pooled. Subsequently, red blood cells were lysed and cells were stained for the indicated lineage and cell surface markers, expression levels were determined for gated (as indicated) cell populations via flow cytometry and compared to the PBS -> Ova (allergy positive control, grey) group, Abbreviations: *No diff.:* no difference to PBS -> Ova group, *n. det.*: no expression detectable, +: up-regulation, –: down-regulation, (+) / (-): weak up- or down-regulation.

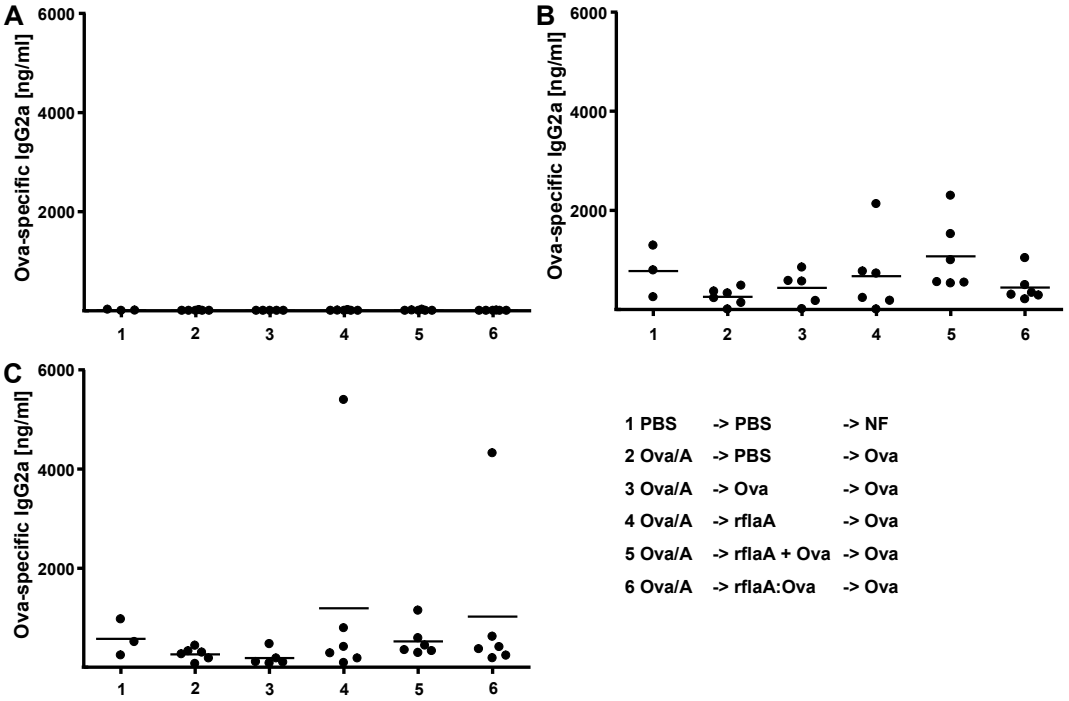
Day 6.5 MLN			PBS -> NF	Ova -> NF	Ova -> Ova	F -> Ova	F +O -> Ova	F:O -> Ova
TC	CD4	PD-1	staining failed	staining failed	staining failed	staining failed	staining failed	staining failed
		CD62-L	++	n. det.	no diff	+	++	no diff
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
	CD8	CD62-L	++	n. det.	no diff	no diff	++	no diff
		CD69	n. det.	n. det.	n. det.	n. det.	n. det.	n. det.
BC	CD19	CD40	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD69	+	no diff.	no diff.	no diff.	no diff.	no diff.
		CD80	++	no diff.	no diff.	no diff.	no diff.	no diff.
		CD86	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC I	-	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC II	no diff.	n. det.	no diff.	-	-	(-)
DC	CD11c	CD40	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD80	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		CD86	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC I	no diff.	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC II	no diff.	n. det.	no diff.	-	(-)	no diff.



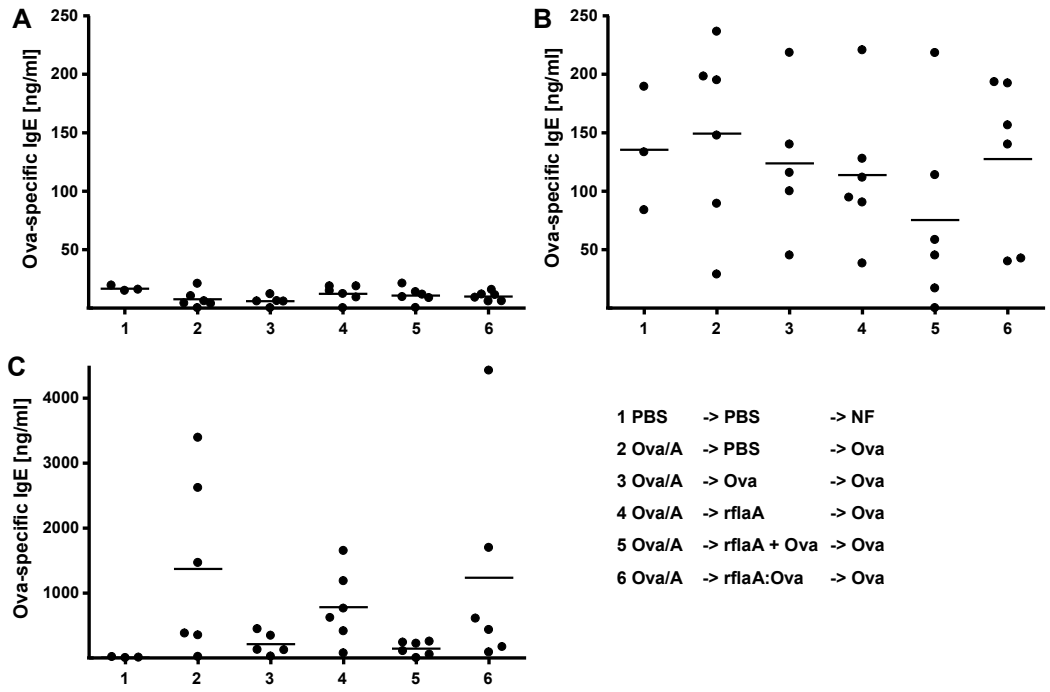
Repository figure 8: Prophylactic vaccination study: Vaccination with rflaA and rflaA:Ova alters T cell activation. On day 6.5 of Ova-pellet challenge animals were sacrificed, mesenteric lymph nodes were prepared, and three animals per group were pooled. Subsequently, red blood cells were lysed and cells were stained for the indicated lineage and cell surface markers, expression levels were determined for gated (as indicated) cell populations via flow cytometry and compared to the PBS -> Ova (allergy positive control, grey) group, Abbreviations: *No diff.:* no difference to PBS -> Ova group, *n. det.*: no expression detectable, +: up-regulation, –: down-regulation, (+) / (-): weak up- or down-regulation.



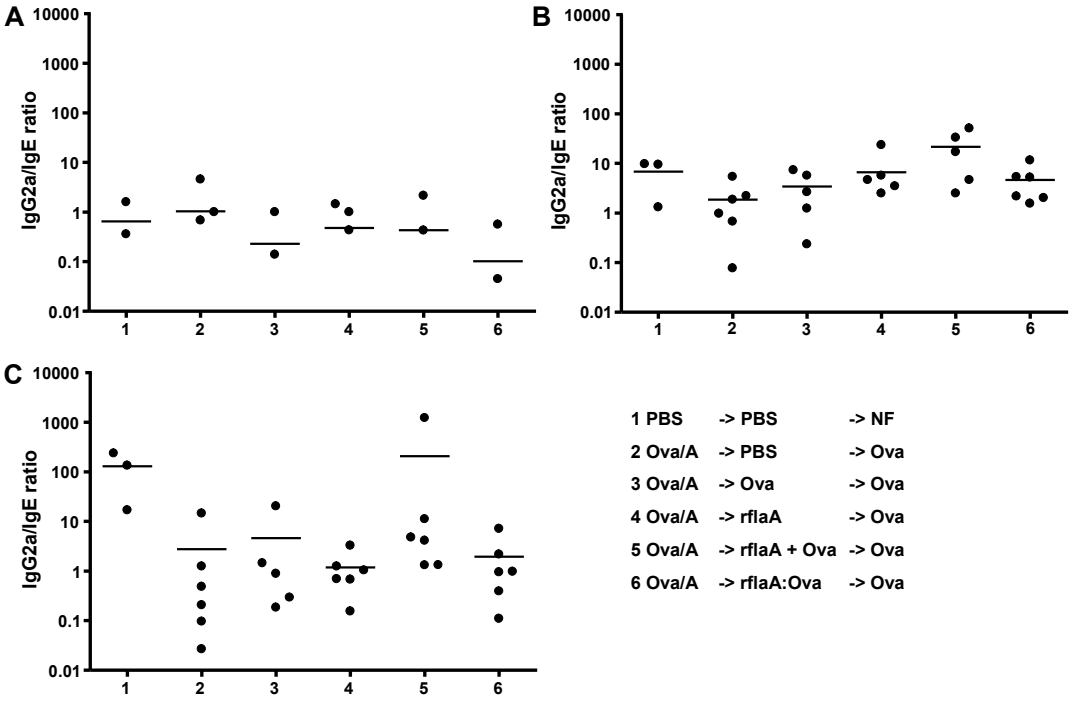
Repository figure 9: Therapeutic vaccination study: Therapeutic vaccination with rflaA:Ova does not induce IgG1 production. Sera of control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were collected one week after the first immunization (A), one week after the second immunization (B), and on the final day of Ova pellet challenge (C). Sera were checked for levels of Ova-specific IgG1 antibodies by ELISA (n = 6 mice per group).



Repository figure 10: Therapeutic vaccination study: Ova-specific IgG2a titers – vaccination with rflaA:Ova does not lead to IgG2a production. Sera of control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were collected one week after the first immunization (A), one week after the second immunization (B), and on the final day of Ova pellet challenge (C). Sera were checked for levels of Ova-specific IgG2a antibodies by ELISA (n = 6 mice per group).

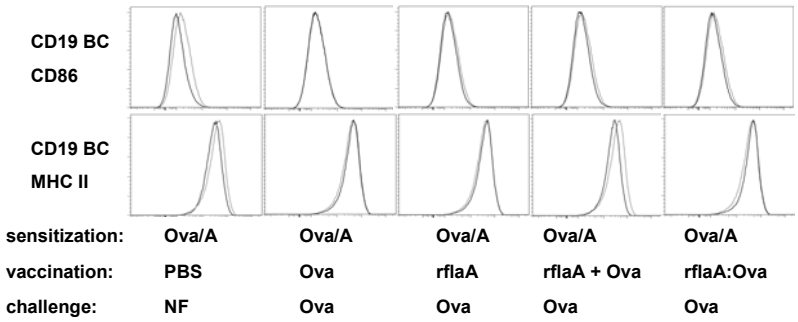


Repository figure 11: Therapeutic vaccination study: Ova-specific IgE titers – vaccination with rflaA:Ova does not suppresses IgE production. Sera of control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were collected one week after the first immunization (A), one week after the second immunization (B), and on the final day of Ova pellet challenge (C). Sera were checked for levels of Ova-specific IgE antibodies by ELISA (n = 6 mice per group).



Repository figure 12: Therapeutic vaccination study: Therapeutic vaccination with rflaA:Ova does not change IgG2a/IgE ratio. Sera of control (PBS -> PBS -> NF) and Ova-sensitized mice (all other groups) were collected one week after the first immunization (A), one week after the second immunization (B), and on the final day of Ova pellet challenge (C). Sera were checked for levels of Ova-specific IgG2a and IgE antibodies by ELISA, IgG2a to IgE ratios were calculated for each mouse and time point (n = 6 mice per group).

Day 5.5 spleen			PBS -> NF	Ova -> Ova	F -> Ova	F +O -> Ova	F:O -> Ova
TC	CD4	PD-1	--	no diff.	-	-	--
		CD62-L	++	no diff.	no diff.	(+)	+
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.
	CD8	CD62-L	++	no diff.	no diff.	(+)	+
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.
BC	CD19	CD40	no diff.	no diff.	no diff.	no diff.	no diff.
		CD69	no diff.	no diff.	no diff.	no diff.	(+)
		CD80	(-)	no diff.	no diff.	no diff.	no diff.
		CD86	-	no diff.	(-)	(-)	(-)
		MHC I	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC II	-	no diff.	no diff.	-	no diff.
DC	CD11c	CD40	(-)	no diff.	(-)	no diff.	no diff.
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.
		CD80	(-)	no diff.	(-)	(-)	(-)
		CD86	(-)	no diff.	(-)	(-)	(-)
		MHC I	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC II	(+)	no diff.	no diff.	no diff.	(-)?

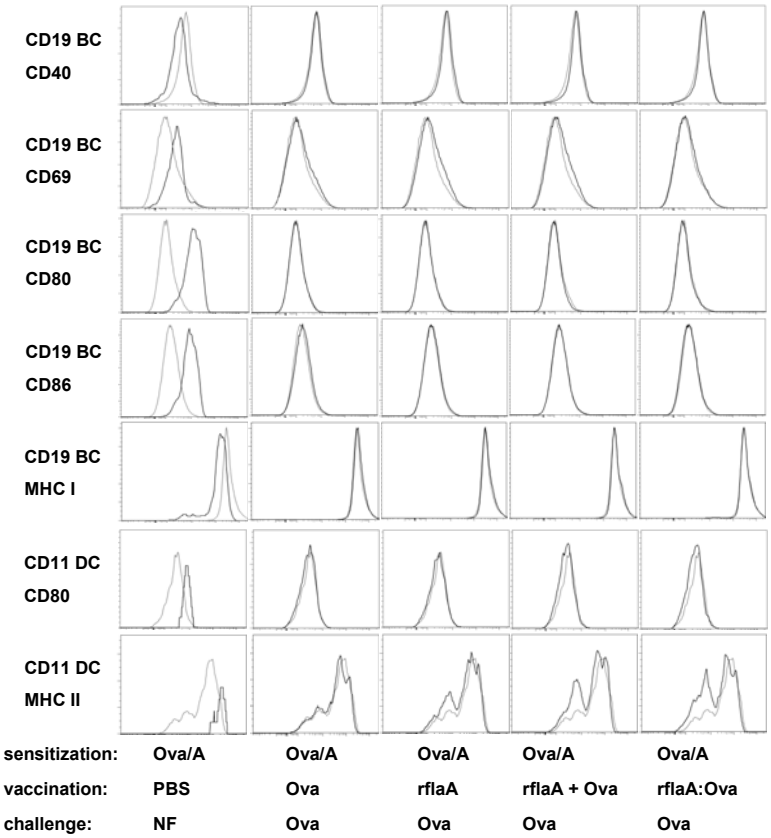


Repository figure 13: Therapeutic vaccination study: Vaccination with rflaA:Ova prevents CD4 and CD8 T cell activation. On day 5.5 of Ova-pellet challenge animals were sacrificed, spleens were prepared, and three animals per group were pooled. Subsequently, red blood cells were lysed and expression levels of the indicated lineage and cell surface markers were determined by flow cytometry, and compared to the PBS -> Ova (allergy positive control, grey) group, Abbreviations: *No diff.:* no difference to PBS -> Ova group, *n. det.*: no expression detectable, +: up-regulation, –: down-regulation, (+) / (-): weak up- or down-regulation.

Day 6.5 spleen			Ova -> Ova	F -> Ova	F +O -> Ova	F:O -> Ova
TC	CD4	PD-1	no data	no data	no data	no data
		CD62-L	+	+	++	+++
		CD69	no diff.	no diff.	no diff.	no diff.
	CD8	CD62-L	+	+	++	+++
		CD69	no diff.	no diff.	no diff.	no diff.
BC	CD19	CD40	no diff.	no diff.	no diff.	no diff.
		CD69	no diff.	no diff.	no diff.	no diff.
		CD80	no diff.	no diff.	no diff.	no diff.
		CD86	no diff.	no diff.	no diff.	no diff.
		MHC I	no diff.	no diff.	no diff.	no diff.
		MHC II	no diff.	no diff.	no diff.	(-)
DC	CD11c	CD40	no diff.	no diff.	no diff.	no diff.
		CD69	no diff.	no diff.	no diff.	no diff.
		CD80	no diff.	no diff.	no diff.	no diff.
		CD86	no diff.	no diff.	no diff.	no diff.
		MHC I	no diff.	no diff.	no diff.	no diff.
		MHC II	no diff.	no diff.	no diff.	no diff.

Repository figure 14: Therapeutic vaccination study: rflaA:Ova-vaccination reduces TC activation. On day 6.5 of Ova-pellet challenge animals were sacrificed, spleens were prepared, and three animals per group were pooled. Subsequently, red blood cells were lysed and expression levels of the indicated lineage and cell surface markers were determined by flow cytometry, and compared to the PBS -> Ova (allergy positive control) group, Abbreviations: *No diff.:* no difference to PBS -> Ova group, *n. det.*: no expression detectable, +: up-regulation, –: down-regulation, (+) / (-): weak up- or down-regulation.

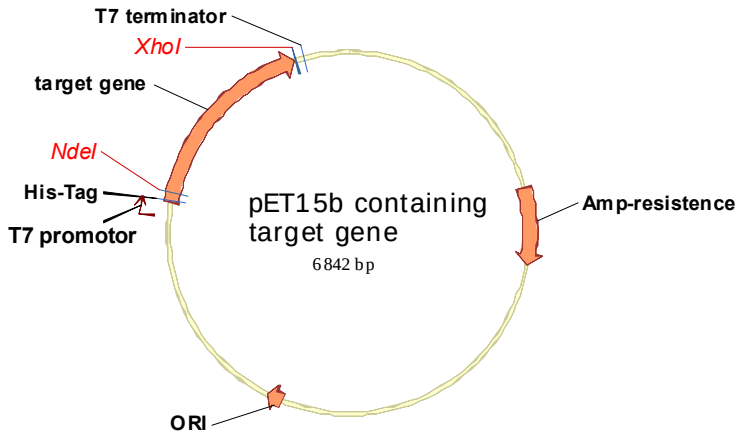
Day 5.5 MLN			PBS -> NF	Ova -> Ova	F -> Ova	F +O -> Ova	F:O -> Ova
TC	CD4	PD-1	+?	-	-	-	-
		CD62-L	no diff.	no diff.	no diff.	+	++
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.
	CD8	CD62-L	++	no diff.	no diff.	(+)	+
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.
BC	CD19	CD40	-	no diff.	no diff.	no diff.	no diff.
		CD69	+	no diff.	no diff.	no diff.	no diff.
		CD80	++	no diff.	no diff.	no diff.	no diff.
		CD86	++	no diff.	no diff.	no diff.	no diff.
		MHC I	-	no diff.	no diff.	no diff.	no diff.
		MHC II	(-)	no diff.	no diff.	no diff.	no diff.
DC	CD11c	CD40	no diff.	no diff.	no diff.	no diff.	no diff.
		CD69	no diff.	no diff.	no diff.	no diff.	no diff.
		CD80	+	no diff.	no diff.	no diff.	no diff.
		CD86	(+)	no diff.	no diff.	no diff.	no diff.
		MHC I	no diff.	no diff.	no diff.	no diff.	no diff.
		MHC II	+	no diff.	-	-	-



Repository figure 15: Therapeutic vaccination study: Therapeutic vaccination affects many parameters in the mesenteric lymph nodes. On day 5.5 of Ova-pellet challenge animals were sacrificed, mesenteric lymph nodes were prepared, and three animals per group were pooled. Subsequently, red blood cells were lysed and expression levels of the indicated lineage and cell surface markers were determined by flow cytometry, and compared to the PBS -> Ova (allergy positive control, grey) group, Abbreviations: *No diff.:* no difference to PBS -> Ova group, *n. det.*: no expression detectable, +: up-regulation, –: down-regulation, (+) / (-): weak up- or down-regulation.

Day 6.5 MLN			Ova -> Ova	F -> Ova	F +O -> Ova	F:O -> Ova
TC	CD4	PD-1	no data	no data	no data	no data
		CD62-L	+	+	++	+++
		CD69	no diff.	no diff.	no diff.	no diff.
	CD8	CD62-L	+	+	++	+++
		CD69	no diff.	no diff.	no diff.	no diff.
BC	CD19	CD40	no diff.	no diff.	no diff.	no diff.
		CD69	no diff.	no diff.	no diff.	no diff.
		CD80	no diff.	no diff.	no diff.	no diff.
		CD86	no diff.	no diff.	no diff.	no diff.
		MHC I	no diff.	no diff.	no diff.	no diff.
		MHC II	no diff.	no diff.	no diff.	no diff.
DC	CD11c	CD40	no diff.	no diff.	no diff.	no diff.
		CD69	no diff.	no diff.	no diff.	no diff.
		CD80	no diff.	no diff.	no diff.	no diff.
		CD86	no diff.	no diff.	no diff.	no diff.
		MHC I	no diff.	no diff.	no diff.	no diff.
		MHC II	(-)	(-)	(-)	(-)

Repository figure 16: Therapeutic vaccination study: rflaA:Ova-vaccinated animals show a reduced TC activation in MLN. On day 6.5 of Ova-pellet challenge animals were sacrificed, mesenteric lymph nodes were prepared, and three animals per group were pooled. Subsequently, red blood cells were lysed and expression levels of the indicated lineage and cell surface markers were determined by flow cytometry, and compared to the PBS -> Ova (allergy positive control, grey) group, Abbreviations: *No diff.:* no difference to PBS -> Ova group, *n. det.*: no expression detectable, +: up-regulation, –: down-regulation, (+) / (-): weak up- or down-regulation.



Repository figure 17: Vector chart for expression of the different constructs using pET15b. All target genes were cloned into the depicted pET15b plasmid using NdeI and XhoI restriction sites. Amp-resistance = Ampicilling resitance gene; *ORI = origin of replication.*

9. Curriculum vitae

Personal data

Name	Schülke
First name	Stefan
Address	Brucknerstraße 35, 64291 Darmstadt
Date of birth	29.01.1983
Place of birth	Bad Kreuznach

Studies and graduation

since 11/2007	PhD-thesis at the Paul-Ehrlich-Institut in Langen Division of Allergology Title of dissertation: „Flagellin:allergen fusion proteins as novel vaccines for the treatment of severe type I allergies “ Supervisor: Prof. Dr. Stefan Vieths Degree: Dr. rer. nat. Mark: result pending
04/03 to 10/07	Studies of biology at the Johannes Gutenberg-Universität in Mainz Main focus: immunology, molecular genetics, zoology and biophysics Diploma thesis at the institute of molecular genetics Title of thesis: „Molecular analysis of genes involved in ROS defence in the hypoxia-tolerant blind mole rat *Spalax“* Supervisor: Prof. Dr. Thomas Hankeln Degree: diplome biologist Mark: excellent

Advanced training

09/09	Successful participation in advanced training: „Gentechnik-Projektleiter und Beauftragten für biologische Sicherheit“, Johannes Gutenberg-Universität in Mainz
07/08	Laboratory animal science - FELASA category B course, Johannes Gutenberg-Universität in Mainz

Further work experience

10/07	Practical course in the institute for Neurochirurgische Pathophysiologie, Johannes Gutenberg-Universität medical clinic in Mainz
05 to 07	Work as research assistant for the institutes of genetics and molecular genetics, Johannes Gutenberg-Universität in Mainz

Scholarships and awards

01/11	Scholarship of the 9th EAACI-GA^{2}LEN-Immunology Winter School
10/10	Poster prize 4th International Symposium on Molecular Allergology
02/10	Scholarship of the 8th EAACI-GA^{2}LEN-Immunology Winter School
04/07 to 09/07	Scholarship of the Johannes Gutenberg-Universität in Mainz

Military service

07/02 to 03/03	For the Fernmeldebataillon 282, Hunsrückkaserne Kastellaun

Education

08/93 to 03/02	Gymnasium am Römerkastell, Bad Kreuznach degree: Abitur Mark: 1,3

Voluntary work

04/96 to 12/01	Work for the „Katholisch-Öffentliche “ library in Guldental. Fields of activity: stockpiling, portfolio maintenance, customer service, planning, organization, and execution of book exhibitions

Further qualifications

Foreign languages	German: mother tongue English: very good skills French: good skills
Computer literacy	Profound knowledge of all Microsoft office applications (Word, Outlook, Powerpoint, Excel), as well as multiple graphics-, analysis-, and bioinformatic programs

Hobbies

	literature (science-fiction) sports (running, boxing) films

Langen, 25.01.11

(Stefan Schülke)

10. Project related publication list

Peer-reviewed

Schülke Stefan[#], Waibler Zoe[#], Mende Marc-Stefan, Zoccatelli Gianni, Vieths Stefan, Masako Toda, Scheurer Stephan ([#] equally contributed). Fusion protein of TLR5-ligand and allergen potentiates activation and IL-10 secretion in murine myeloid DC. *Mol.Immunol. (2010), doi:10.1016/j.molimm.2010.07.006.*

Abstracts and non Peer-reviewed publications

Schülke Stefan, Burggraf Manja, Waibler Zoe, Wangorsch Andrea, Kalinke Ulrich, Toda Masako, Vieths Stefan, Scheurer Stephan. Recombinant TLR5-ligand flagellin:Ova fusion protein prevents intestinal allergy in mice (accepted oral presentation). *World Immune Regulation Meeting, WIRM V, Davos, Switzerland, 24 - 27 March 2011*.

Schülke Stefan, Burggraf Manja, Waibler Zoe, Wangorsch Andrea, Kalinke Ulrich, Toda Masako, Vieths Stefan, Scheurer Stephan. Recombinant TLR5-ligand flagellin:Ova fusion protein prevents intestinal allergy in mice (travel grant and oral presentation). *9th EAACI-Ga²len Immunology Winter School, Davos, Switzerland, 03 – 06 February 2011.*

Schülke Stefan, Waibler Zoe, Wangorsch Andrea, Wolfheimer Sonja, Kalinke Ulrich, Burggraf Manja, Toda Masako, Vieths Stefan, Scheurer Stephan. Recombinant flagellin:Ova fusion protein enhances IL-10 secretion from mDC and represses Th1/Th2 immune responses (poster presentation). *4th International Symposium on Molecular Allergology (ISMA 2010), Munich, Germany, 29 – 31 Oktober 2010.*

Schülke Stefan, Waibler Zoe, Wangorsch Andrea, Wolfheimer Sonja, Kalinke Ulrich, Burggraf Manja, Toda Masako, Vieths Stefan, Scheurer Stephan. Recombinant TLR5-ligand flagellin:Ova fusion protein enhances IL-10 secretion from mDC, represses Th1/Th2 immune responses in vitro, and prevents intestinal allergy in vivo (oral presentation). *4th International Symposium on Molecular Allergology (ISMA 2010), Munich, Germany, 29 – 31 Oktober 2010.*

Schülke Stefan, Wangorsch Andrea, Waibler Zoe, Kalinke Ulrich, Toda Masako, Vieths Stefan, Scheurer Stephan. Recombinant TLR5-ligand flagellin:Ova fusion protein enhances IL-10 secretion from mDC and represses Th1/Th2 immune response *in vitro* (poster presentation). *World Immune Regulation Meeting, WIRM IV, Davos, Switzerland, 29 March – 01 April 2010*.

Bohnen Christof, Albrecht Melanie, Reese Gerald, Toda Masako, Schülke Stefan, Schwantes Astrid, Süzer Yasemin, Sutter Gerd, Scheurer Stephan, Vieths Stefan. Recombinant Modified Vaccinia Virus Ankara as an Allergy Vaccine Candidate (poster presentation). *World Immune Regulation Meeting, WIRM IV, Davos, Switzerland, 29 March – 01 April 2010*.

Schülke Stefan, Wangorsch Andrea, Waibler Zoe, Kalinke Ulrich, Toda Masako, Vieths Stefan, Scheurer Stephan. Recombinant TLR5-ligand flagellin:Ova fusion protein enhances IL-10 secretion from mDC and represses Th1/Th2 immune response *in vitro* (oral presentation). *22. Mainzer Allergie Workshop, Mainz, Germany, 11 – 12 March 2010.*

Bohnen Christof, Albrecht Melanie, Reese Gerald, Toda Masako, Schülke Stefan, Schwantes Astrid, Süzer Yasemin, Sutter Gerd, Scheurer Stephan, Vieths Stefan. Recombinant Modified Vaccinia Virus Ankara (MVA) as a novel vaccine candidate for the prevention of allergy (oral presentation). *22. Mainzer Allergie Workshop, Mainz, Germany, 11 – 12 March 2010.*

Hendrik Wiesner, Stefan Schülke, Stephan Scheurer, Viola Kohlrautz, Eckard Hamelmann, Ulrich Wahn, Philippe Stock. Tierexperimentell nachgewiesene Induktion einer Immuntoleranz durch hitzeinaktivierte Listerien - therapeutische Strategie auch beim Menschen? (poster presentation). *GPP-Kongress Berlin 2010, Poster Session AG Experimentelle Pneumologie, Berlin, Germany, 4 March 2010.*

Schülke Stefan, Wangorsch Andrea, Waibler Zoe, Kalinke Ulrich, Toda Masako, Vieths Stefan, Scheurer Stephan. Recombinant TLR5-ligand flagellin:Ova fusion protein enhances IL-10 secretion from mDC and represses Th1/Th2 immune response *in vitro* (travel grant and oral presentation). *8th EAACI-Ga²len Immunology Winter School, Grainau, Germany, 11 – 14 February 2010.*

Hendrik Wiesner, Stefan Schülke, Stephan Scheurer, Viola Kohlrautz, Eckard Hamelmann, Ulrich Wahn, Philippe Stock. Induction of allergen-specific immunotolerance by the use of heat-killed-*Listeria monocytogenes* and flagellin as adjuvants (travel grant and poster presentation). *8th EAACI-Ga²len Immunology Winter*
School, Grainau, Germany, 11 – 14 February 2010.

Schülke Stefan, Mende Stefan, Zoccatelli Gianni, Toda Masako, Waibler Zoe, Vieths Stefan, Scheurer Stephan. Immune modulating properties of a recombinant allergen conjugated with the TLR5-ligand flagellin (poster presentation). *World Immune Regulation Meeting, WIRM III, Davos, Switzerland, 22 – 25 March 2009.*

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